Removal of Syndecan-1 Promotes TRAIL-Induced Apoptosis in Myeloma Cells

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Syndecan-1 is the major transmembrane proteoglycan in cells. Of the four syndecans, syndecan-1 is the dominant form expressed in multiple myeloma and is an indicator of poor prognosis. In the current study, we observed that early TRAIL-induced apoptotic processes were accompanied by cleavage of syndecan-1 intracellular region, and explored the possibility whether removal of syndecan-1 promotes apoptotic processes. We found that syndecan-1 knockdown by specific small interfering RNA in multiple myeloma enhanced TRAIL-induced apoptosis, even though the expression of TRAIL receptors and several apoptosis-associated molecules was unaffected. The enhanced TRAIL-mediated apoptosis in syndecan-1–deficient cells was not due to a decrease in surface heparan sulfate or a reduction in TRAIL receptor endocytosis. The increase in TRAIL-induced cell death was accompanied by an elevated caspase-8 activation and an enhanced formation of death-inducing signaling complexes, which could be attributed to an increased expression of TRAIL receptor O-glycosylation enzyme in syndecan-1–deficient cells. We also found that in H9 lymphoma and Jurkat cells, knockdown of the predominant syndecan member also led to an increase in Fas ligand-induced apoptosis. Our results demonstrate that syndecan plays a negative role in death receptor-mediated cell death, suggesting potential application of syndecan downregulation in the treatment of myeloma in combination with TRAIL. The Journal of Immunology, 2012, 188: 2914–2921.

Syndecan-1 also regulates the activation of α5β1 and αvβ3 integrins in angiogenesis (13).

Engagement of Fas and TRAIL receptors (death receptor DR) by the Fas ligand (FasL) and TRAIL, respectively, leads to formation of death-inducing signaling complexes (DISC) containing Fas-associated protein with death domain (FADD) and procaspase-8 at the cell membrane (14–16). For the TNF-α receptor, the formation of DISC does not occur at the cell membrane: TNFR type 1-associated death domain protein (TRADD) has to be dissociated from membrane-bound TNFRI complex, and FADD and procaspase-8 become then associated with TRADD in the cytoplasm (16–19). DISC is serving as a platform for procaspase-8 to undergo autoproteolytic cleavage generating active caspase-8. Caspase-8 in turn activates downstream caspases and leads to irreversible cell damage. DRs like Fas are associated with actin filaments through binding to ezrin/radixin/moesin (20). Syndecan binds directly to the actin cytoskeleton (21) and is indirectly linked to Fas.

Since the identification of Fas and TRAIL receptors, the possibility of DR agonists to induce apoptosis in tumor cells has been intensively explored. TRAIL and TRAIL receptor agonists trigger apoptosis in tumor cells while leaving normal cells unaffected, and are promising biologic drugs for anticancer therapy, shown by results from many clinical trials (22–26). A few cancer cells are relatively resistant to TRAIL, and TRAIL therapies often involve sensitization of the transformed cells to TRAIL-induced apoptosis. Myeloma cells are moderately responsive to TRAIL (27–29). In the current study, we observed that TRAIL-induced apoptosis is preceded by removal of the intracellular domain of syndecan-1 in myeloma cells, and investigated in this study how TRAIL-induced apoptosis is modulated by syndecan-1 removal. We found that syndecan-1 knockdown leads to a significant increase in the formation of DISC and in TRAIL-induced cell death in myeloma cells. In addition, downregulation of the major form of syndecan in T lymphoma cells enhanced FasL-induced apo-
ptosis. Our results illustrate a negative role of syndecan in TRAIL- and FasL-induced apoptosis, and suggest that knockdown of syndecan may be used to increase the efficacy of TRAIL in targeting myeloma.

Materials and Methods

Reagents

Recombinant human TRAIL and anti-His were obtained from R&amp;D Systems (Minneapolis, MN). FLAG-TRAIL, His-TRAIL, FasL- and anti-c-FLIP (NF6) were purchased from ENZO Life Sciences (Plymouth Meeting, PA). Anti–syndecan-1 (DL-101), anti–syndecan-3 (M-300), anti–syndecan-4 (5G9), anti–DR4 (H-130), anti–Fas (C-20), anti–Mcl-1 (S-19), anti–Bel-2 (N-19), anti–FADD (H-181), and anti–caspase-3 (H-277) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–syndecan-1 (BV-600) and annexin V-Cy5 were obtained from Biovision (Mountain View, CA). Anti-human syndecan-1 (B-B4) was purchased from Sigma-Aldrich (St. Louis, MO). Anti–caspase-8 (18C8), and anti–active caspase-3 were purchased from Cell Signaling (Beverly, MA). Allophycocyanin-conjugated anti-human DR4 (DJR1), allophycocyanin anti-DR5 (DJR2-4), DyLight649 anti–rabbit IgG, and allophycocyanin anti-mouse IgG were obtained from BioLegend (San Diego, CA). Anti–β-tubulin (clone A2A) was purchased from Upstate (Lake Placid, NY). The STSN/95-115 peptide (80-90% purity) was purchased from GenScript (Piscataway, NJ). Goat anti–rabbit Ig and rabbit anti–mouse Ig conjugated with HRP were obtained from Amersham Biosciences (Buckinghamshire, U.K.). Rabbit anti–syndecan-2 was a gift of Y-P. Hsueh (Institute of Molecular Biology, Academia sinica).

Cell culture and apoptosis

Cells were cultured in RPMI 1640 media with 10% FCS (Life Technologies-Invitrogen), 10 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml 2-ME. Two different methods were used for apoptosis measurements after DR ligand treatments. Cells were stained with allophycocyanin annexin V, and annexin V+ populations were quantified by flow cytometry. Cells were also stained with propidium iodide (PI) in hypotonic solution (50 μg/ml PI, 0.1% sodium citrate, 0.1% Triton X-100) overnight at 4˚C. Fractions of cells with sub-G1 DNA content were determined using the CELLFIT program on a FACS Calibur flow cytometer (BD Biosciences, Mountain View, CA). All apoptosis events were confirmed by both measurements.

Syndecan knockdown

Syndecan-1 was downregulated by two different methods. Multiple myeloma cells (5 × 10⁶) were transfected with syndecan-1–specific small interfering RNA (siRNA) by electroporation on an MP-100 Microparator (Digital Bio) at 1600 V in using two pulses of 15-μs duration, and cellular syndecan-1 was analyzed 48 h later. The sequence of siRNA for syndecan-1 was 5’-CAC CUG GCA UCG CAC CAU U-3’. Alternatively, syndecan-1, syndecan-2, or syndecan-4 knockdown lentiviral constructs were produced by incorporating syndecan-1–, syndecan-2–, or syndecan-4–specific short hairpin RNA (shRNA) sequence into the plLentiLox vector (pL3.7; obtained from I-C. Ho, Harvard Medical School, Boston, MA). The following shRNA sequences were employed: short hairpin syndecan-1 (shSDC1): 5’-TTG GTC AGC CTC TA-3; shSDC2: 5’-CCT TGA GGT TGT TAC CTT GAA CTA-3; shSDC4: 5’-GGT GAG GTG GTC CTC TCA ATA-3; and shSDC5: 5’-GGT GAG GTG GTC CTC TCA ATA-3. Lentiviruses were harvested from cell cultures of 293FT cells transfected with pL3.7 or pL3.7-shSDC4 (20 μg), pSPAX2 (15 μg), and pMD2.G (6 μg). H929 cells, RPMI 8226, U266, H9, or Jurkat cells were infected with recombinant lentiviruses, and GFP-expressing cells were isolated by sorting on a FACS Vantage SE system (BD Biosciences) 48 h postinfection.

Overexpression of syndecan-1

Human syndecan-1 cDNA was subcloned into pTRIP-IRES-GFP to generate pTRIP-SDC-1-IRES-GFP. The 293T cells were transfected with 20 μg pTRIP-IRES-GFP or pTRIP-SDC-1-IRES-GFP, 15 μg psPAX2, and 6 μg pMD2.G, and lentivirus-containing culture supernatants were harvested. Cells were infected with recombinant lentivirus with pTRIP-IRES-GFP or pTRIP-SDC-1-IRES-GFP, and GFP-expressing cells were isolated 48 h postinfection by sorting on a FACS Vantage SE.

Surface staining

For staining of surface syndecans, cells were incubated with anti–syndecan-1, anti–syndecan-2, anti–syndecan-3, or anti–syndecan-4 in PBS containing 1% FBS. After washing with PBS, cells were stained with allophycocyanin-conjugated anti-mouse IgG or DyLight649 anti-rabbit IgG, and analyzed on a FACS Calibur flow cytometry system. For TRAIL receptor internalization, H929 and RPMI 8226 cells were incubated with FLAG-tagged TRAIL (2 μg/ml) on ice for 30 min, followed by incubation at 37˚C for 5, 10, or 30 min. Receptor internalization was stopped by adding ice-cold PBS containing 0.5% sodium azide. Cells were then washed with ice-cold PBS and stained with anti-FLAG, followed by allophycocyanin-labeled anti-mouse IgG for FACS analysis.

Caspase-8 activity determination

Caspase-8 activity was quantitated by the Caspase-Glo 8 assay (Promega, Madison, WI). Myeloma cells were treated with TRAIL for 1.5 h, and an equal volume of Caspase-Glo 8 reagent was then added and incubated for additional 30 min. The aminoluciferin released by caspase-8-mediated cleavage of precursor was determined using a luminescence reader Victor3 1420 Multilabel Counter (PerkinElmer, Shelton, CT).

DISC immunoprecipitation

Aliquots of 2 × 10⁶ H929 cells were incubated with His-TRAIL (5 μg/ml) for 30 min and then at 37˚C for 30 or 60 min. Cells were lysed in DISC immunoprecipitation buffer (30 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 10% glycerol, and protease inhibitors [Roche, Mannheim, Germany]). Cell lysates were incubated with Ni-NTA agarose (Qiagen, Hilden, Germany) for 2 h. DISC complexes were eluted from Ni-NTA agarose by 200 μM imidazole. The eluent was resolled by SDS-PAGE and probed with Abs for DR4, DR5, caspase-8 (Cell Signaling), FADD, and β-tubulin. DISC precipitation in H9 cells using FLAG-FasL and anti-FLAG beads was performed, as previously described (30).

For detection of TRAIL receptor clustering, 2 × 10⁵ cells were incubated with human rTRAIL (200 ng/ml; R&amp;D Systems) for the indicated times. Cells were lysed in DISC immunoprecipitation buffer. A total of 2 μl cell lysates were incubated overnight with 2 μg anti–caspase-8 (C-20; Santa Cruz Biotechnology) preloaded on 20 μl protein G-Sepharose at 4˚C. The precipitants were washed and resolved in 4-20% nonreducing SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membrane was probed with anti–caspase-8 (1C12; Cell Signaling) and anti-DR4 (Santa Cruz Biotechnology).

Quantitative PCR

Total RNA from control and syndecan-1 knockdown H929 cells was isolated using TRizol (Invitrogen). cDNAs were synthesized and analyzed for the expression of GALNT3 and GALNT14 on a LightCycler 480 Real-Time PCR System (Roche). The PCR protocol is 95˚C for 10 min, followed by 45 cycles of 95˚C for 10 s, 60˚C annealing for 10 s, and 72˚C extension for 8 s. The PCR primers are as follows: GALNT3, forward, 5’-AAA GCG TTG GTC AGC CTC ATA-3’ and reverse, 5’-AAC GAG ACC TGT AGC AGC AT-3’; GALNT14, forward, 5’-CTG AGA TGC ACA CTG CTG GT-3’ and reverse, 5’-CAT TTC ACC TTG GAC AAC TT-3’.

Results

Processing of syndecan-1 occurs early during TRAIL-induced apoptosis in myeloma cells

Syndecan-1 was the most abundant syndecan on the surface of multiple myeloma H929, RPMI 8228, and U266 cells (Supplemental Fig. 1A, not shown for U266). We found that treatment with TRAIL was accompanied with the appearance of a low molecular mass (<15 kDA) fragment of syndecan-1, as detected by immunoblot analysis of H929 cells (Fig. 1A). The generation of the syndecan-1 fragment coincided with the activation of caspase-8 (Fig. 1A). Further support that the cleavage of syndecan-1 was mediated by caspase was obtained by the complete blockade of the processing by the addition of Z-VAD, a cell-permeable caspase inhibitor (Fig. 1C). In addition, the cleavage of syndecan-1 was partially prevented by the caspase-8 inhibitor Z-IETD, indicating an involvement of caspase-8. Notably, there was no apparent reduction in the surface level of syndecan-1.
Downregulation of syndecan-1 increases the sensitivity to TRAIL-induced apoptosis in myeloma cells

To explore the possibility that removal of syndecan-1 intracellular domain enhances DR-mediated cell death, syndecan-1 was knocked down in these myeloma cell lines by transfection with syndecan-1-specific siRNA (siSDC1). Fig. 2A illustrates that syndecan-1 was nearly eliminated in H929 cells transfected with siSDC1. This was accompanied by diminished expression of surface syndecan-1, with a reduction by >90% (Fig. 2B). Syndecan-1 knockdown did not affect the growth and viability of myeloma cells in vitro (data not shown), similar to a previous report (12). Syndecan-1 was similarly downregulated in RPMI 8226 myeloma

(Fig. 1B), in contrast to the disappearance of syndecan-1 in cell lysates 90 min after TRAIL treatment (Fig. 1C, DMSO). Because different syndecan-1Abs were used for Western blotting (BV-600) and FACS staining (clone DL-101), we investigated whether the Ab used for immunoblotting detected the C-terminal portion of syndecan-1 and whether the low molecular mass fragment represented the intracellular part of syndecan-1. To this end, syndecan-1 with a Myc tag attached to the C-terminal end of syndecan-1 was expressed in H929 cells. Treatment of cells expressing syndecan-1-Myc with TRAIL led to the generation of the low molecular mass fragment detected by anti-Myc (Fig. 1D). These results suggest that immediately following TRAIL receptor ligation, the intracellular region of the major transmembrane glycosaminoglycan was cleaved by early-activated caspases.
cells. The effectiveness of syndecan-1 knockdown was demonstrated by immunoblotting of cell lysates from RPMI 8226 cells (Fig. 2E).

The multiple myeloma cell lines varied with regard to their susceptibility to DR ligands. In agreement with previous observations that myeloma cells are modestly sensitive to TRAIL (27–29), TRAIL triggered a moderate extent of apoptosis in control H929 and RPMI 8226 cells (Fig. 2C, 2D, 2F). By contrast, these myeloma cell lines, which express very low levels of Fas, were refractory to FasL engagement (data not shown). Downregulation of syndecan-1 significantly increased the extent of apoptosis induced by TRAIL in H929 cells, as assessed by both annexin V staining and measurement of sub-G, DNA content (Fig. 2C, 2D). TRAIL-induced apoptosis was also enhanced in syndecan-1-deficient RPMI 8226 cells relative to their control (Fig. 2F). In separate experiments, syndecan-1 was also effectively downregulated by transduction of lentivirus-containing syndecan-1-specific shRNA in H929, RPMI 8226, and U266 cells (Fig. 2G, data not shown). Treatment with TRAIL promoted the apoptosis in these myeloma cell lines relative to the vector control (data not shown). In control experiments, shSDC1-mediated downregulation of syndecan-1 in H9 T lymphoma cells, which minimally expresses this protein (Supplemental Fig. 1B), did not affect TRAIL-induced cell death (data not shown).

TNF-α stimulation triggered a moderate cell death in RPMI 8226 cells (Fig. 2H), similar to a previous report (27), but did not affect the viability of H929 and U266 cells (data not shown). Knockdown of syndecan-1 did not affect TNF-α–induced apoptosis in RPMI 8226 cells (Fig. 2H). Taken together, our results indicate that decreased syndecan-1 expression leads to an increase in TRAIL– but not TNF-α–triggered death in multiple myeloma cells.

Syndecan-1 overexpression inhibits TRAIL-induced apoptosis

We also examined whether syndecan-1 overexpression in H929 cells also affected TRAIL-mediated cell death. H929 cells were infected with recombinant lentivirus for syndecan-1 expression. The elevated syndecan-1 expression was confirmed by flow cytometry (Fig. 3A). Increased expression of syndecan-1 in H929 cells suppressed TRAIL-induced apoptosis (Fig. 3B), consistent with a negative role of syndecan-1 in TRAIL-initiated cell death in myeloma cells.

Normal expression of DR4/DR5 and apoptosis-associated molecules in syndecan-1 knockdown myeloma cells

Enhanced TRAIL sensitivity in some cancer cells has been linked to an increase in surface TRAIL receptors (22–26). The expression of total TRAIL receptors, as determined by labeling with FLAG-TRAIL, was not altered in syndecan-1 knockdown H929 cells (Fig. 4A). Neither was any increase in the surface level of DR4 or DR5 detectable in syndecan-1 knockdown H929 cells (Fig. 4B). The total cellular levels of DR4 or DR5, as measured by immunoblotting, were comparable in control and syndecan-1–downregulated H929 cells (Fig. 4C). Downregulation of syndecan-1 also did not affect the levels of cell surface and total DR4 and DR5 in RPMI 8226 cells (Fig. 4D, 4E). The observed enhanced TRAIL sensitivity in syndecan-1 knockdown myeloma cells is therefore not associated with any change in the expression of DR4 and DR5.

As the major transmembrane proteoglycan, syndecan-1 transduces signals (5–7) and modulates the expression of apoptosis-associated molecules. c-FLIPL is an anti-apoptotic protein linked to resistance of cancer cells to TRAIL (22–26). Increased Bim expression and reduced Mcl-1 expression have been observed in myeloma cells deficient in Blimp-1 (31), the molecule that regulates syndecan-1 expression. We determined whether syndecan-1 downregulation affected the levels of these apoptosis-related proteins. The expression of FADD, caspase-8, c-FLIPL, Bcl-2, Bim, Bax, and Mcl-1 was not altered in syndecan-1 knockdown H929 cells (Fig. 4C) and syndecan-1–deficient RPMI 8226 cells (Fig. 4E). Therefore, the increase in TRAIL–triggered apoptosis in syndecan-1–deficient myeloma cells cannot be attributed to a change in the expression of these proteins.

Effect of syndecan-1 knockdown in TRAIL-mediated apoptosis is independent of heparan sulfate or integrin association

It has been shown that a reduction in the cell surface heparan sulfate proteoglycan decreases the viability of myeloma in vivo (11, 32). Thus, the increased sensitivity to TRAIL-mediated apoptosis could be due to a diminished quantity of cell surface heparan sulfate in syndecan-1 knockdown cells. To examine this possibility, H929 cells were treated with heparinase III. The removal of heparan sulfate was confirmed by the reduction of the apparent molecular mass of syndecan-1 from 230 kDa to that of the 75-kDa core protein (Supplemental Fig. 2A, 2C). In contrast to syndecan-1 knockdown H929 cells, TRAIL–induced cell death was decreased in heparinase III–treated H929 cells (Supplemental Fig. 2B). Similarly, TRAIL–triggered apoptosis was reduced in heparinase–treated RPMI 8226 cells (Supplemental Fig. 2D). Removal of heparan sulfate proteoglycan therefore inhibits the capacity of TRAIL to induce apoptosis in myeloma cells. These results suggest that enhanced apoptosis observed in syndecan-1–deficient myeloma cells was not due to a reduction in cell surface heparan sulfate.

We also explored the possibility that the increased ability of TRAIL to induce apoptosis is associated with a reduced binding of syndecan-1 to integrin (13). To this end, we used the peptide inhibitor synstatin that has been previously shown to disrupt the interaction between syndecan-1 and integrin. We found that synstatin did not have any effect on TRAIL–triggered apoptosis in H929 and RPMI 8226 cells (Supplemental Fig. 2E, 2F). Therefore, TRAIL–induced cell death in myeloma cells is independent of integrin association, and increased TRAIL–mediated apoptosis

FIGURE 3. Syndecan-1 overexpression inhibits TRAIL-induced apoptosis in H929 cells. (A) Syndecan-1 overexpression in H929 cells. H929 cells were infected with vector alone or pTRIP-SDC1. Cells were sorted based on GFP expression, and cell surface syndecan-1 levels were determined by flow cytometry. Solid line, mock (pTRIP alone); thin line, syndecan-1 transduced (SDC1); shadowed curve, secondary Ab-only control (Ctl). (B) Overexpression of syndecan-1 attenuated TRAIL–triggered cell death. Control and syndecan-1–transduced H929 cells were stimulated with TRAIL at the indicated doses for 6 h, and cell death was determined. **p < 0.01 for paired t test, ***p < 0.001.
cannot be attributed to a diminished interaction with integrin in syndecan-1–deficient cells.

Normal TRAIL receptor endocytosis in syndecan-1–deficient H929 cells

Inhibition of endocytosis of TRAIL receptors has been shown to enhance TRAIL-induced cell death (33, 34). Because syndecan-1 is anchored to cortical actin, we examined whether TRAIL receptor internalization was altered by downregulation of syndecan-1 in H929 cells. FLAG-TRAIL was used to measure the surface levels of TRAIL receptors in H929 cells. Fig. 5A illustrates that TRAIL stimulation led to the disappearance of TRAIL receptors from the cell surface in H929 cells. Knockdown of syndecan-1 did not affect both the degree and the speed of DR4/DR5 endocytosis in H929 cells (Fig. 5A, 5B). Similar kinetics in the endocytosis of TRAIL receptors was also found in RPMI 8226 cells with or without syndecan-1 (Fig. 5C). Therefore, syndecan-1 is not involved in DR4/DR5 internalization, and the increased apoptosis in syndecan-1 knockdown myeloma cells is unrelated to TRAIL receptor endocytosis.

Accelerated caspase-8 activation and enhanced DISC formation in syndecan-1–deficient cells

We measured the biochemical processes following TRAIL stimulation in H929 cells. In syndecan-1 knockdown H929 cells, the cleavage of procaspase-8, the generation of the p43 caspase-8 intermediate, and the emergence of active p18 caspase-8 proceeded earlier than in wild-type H929 cells (Fig. 6A). This was associated with an increased processing of procaspase-3 in syndecan-1–deficient H929 cells (Fig. 6A). We further quantitated caspase-8 activity in these cells. Consistent with an accelerated caspase-8 processing, caspase-8 activity was much higher in syndecan-1 knockdown cells than the control H929 cells stimulated by different doses of TRAIL (Fig. 6B).

Because activation of caspase-8 is among the earliest biochemical events following DR ligation, we examined whether syndecan-1 deficiency affected DISC formation. His-tagged TRAIL was used to immunoprecipitate DISC from H929 cell lysates. Before TRAIL stimulation, His-tagged TRAIL did not pull down FADD or caspase-8 in H929 cells (Fig. 6C). TRAIL treatment led to the assembly of a complex containing caspase-8 and FADD in H929 cells. Syndecan-1 knockdown significantly increased the amounts of FADD and caspase-8 in the DISC (Fig. 6C). Therefore, syndecan-1 knockdown increased TRAIL-induced recruitment of FADD and caspase-8 in H929 cells, leading to accelerated activation of caspase-8 and caspase-3.

Increased N-acetyl galactosamine transferase 3 expression and enhanced receptor clustering in syndecan-1 knockdown H929 cells

Of the several processes that may affect TRAIL receptor DISC formation, we found that the expression of peptidyl N-acetyl galactosamine transferase (GALNT) 3 was increased in syndecan-1 knockdown H929 cells (Fig. 7A). GALNT3 and GALNT14 mediate the O-glycosylation of TRAIL receptors and promote
Increased DISC formation in syndecan-1–downregulated H929 cells. Cell TRAIL.
imidazole and analyzed for the expression of caspase-8, FADD, and His-
immunoprecipitation. DISC in the lysates was pulled down by Ni-NTA agarose. DISC
prepared before (0 min) and 30 or 60 min after His-TRAIL (2
syndecan-1 knockdown H929 cells. (**p < 0.001 for paired t test. (B) Enhanced
caspase-8 activity in syndecan-1 knockdown cells. Control and syndecan-1 knockdown H929 cells were treated with TRAIL for 90 min at the indicated doses. The activity of caspase-8 was determined by the cleavage of Z-LETD aminoluciferin. **p < 0.001 for paired t test. (C) Increased DISC formation in syndecan-1–downregulated H929 cells. Cell lysates of control and syndecan-1–deficient H929 cells (2 × 10^7) were prepared before (0 min) and 30 or 60 min after His-TRAIL (2 μg) treatment. DISC in the lysates was pulled down by Ni-NTA agarose. DISC components were then released from the beads by incubation with 200 mM imidazole and analyzed for the expression of caspase-8, FADD, and His-TRAIL.

TRAIL-induced DR4/DR5 clustering in cancer cell lines, without affecting the surface levels of TRAIL receptors (35). Levels of GALNT3 and GALNT14 mRNA correlate with sensitivity to TRAIL-triggered apoptosis in colorectal cancer and pancreatic cancer cell lines, respectively (35). Consistent with the specific expression of distinct GALNT isoforms in different cancer cells, GALNT14 mRNA was not altered in syndecan-1–deficient H929 cells (Fig. 7A). To evaluate the consequence in TRAIL receptor O-glycosylation, TRAIL-induced clustering of TRAIL receptors was determined in syndecan-1–deficient cells. Immunoprecipitation with caspase-8 revealed enhanced translocation of high molecular mass DR4 into DISC after TRAIL treatment in syndecan-1–downregulated cells (Fig. 7B), in accordance with increase in GALNT3-mediated O-glycosylation of TRAIL receptors.

**Increased Fasl-induced apoptosis in syndecan-4 knockdown H9 cells and syndecan-2–deficient Jurkat cells**

We extended our study on syndecan-1 to other types of cancer cells. We found that for human T lymphoma H9 cells, syndecan-4 was the major form of syndecans on the cell surface (Supplemental Fig. 1B). Syndecan-4 was downregulated by specific shRNA, and the effectiveness of knockdown was confirmed by FACS (Supplemental Fig. 3A). Knockdown of syndecan-4 prominently increased FasL-triggered apoptosis (Supplemental Fig. 3B). By contrast, TRAIL-induced cell death in H929 and U266 cells was not affected by downregulation of syndecan-4 (Supplemental Fig. 4A, 4B). Knockdown of syndecan-4 did not alter the expression of Fas and c-FLIP in H9 cells (Supplemental Fig. 4C, 4D). The enhanced FasL-induced cell death was associated with an accelerated caspase-8 cleavage and elevated level of active p18 caspase-8 (Supplemental Fig. 3C). This was accompanied by a prominent increase in the emergence of active caspase-3. Syndecan-2 is the dominant form of syndecan in the acute T leukemia cell line Jurkat. Syndecan-2 was also knocked down by specific shRNA, as shown by diminished syndecan-2 cell surface levels (Supplemental Fig. 3D). Fasl-triggered apoptosis was significantly increased in syndecan-2 knockdown Jurkat cells (Supplemental Fig. 3E). Because Fas does not contain the O-glycosylation site on TRAIL receptors (35), the increased Fas-initiated cell death in syndecan-4 knockdown H9 and syndecan-2–deficient Jurkat cells is apparently independent of GALNT3 or GALNT14. We examined molecules that specifically modulate Fasl-induced apoptosis. Fas-associated phosphatase-1 (FAP-1) is a negative regulator of Fasl–triggered cell death, and high levels of FAP-1 are correlated with resistance to Fas-mediated apoptosis in selective types of cancer cell (36, 37). The FAP-1 protein levels, however, were comparable between control and syndecan-4 knockdown H9 cells before and after Fasl stimulation (Supplemental Fig. 4E), suggesting that enhanced sensitivity to Fasl-induced apoptosis in syndecan-4–deficient H9 cells is not due to change in FAP-1 levels. These results demonstrate that syndecan also negatively regulates the cell death initiated by Fas, with mechanisms different from how syndecan-1 modulates TRAIL sensitivity.

**Discussion**

In this study, we examined the roles of syndecan in DR-mediated cell death. Because syndecan-1, syndecan-4, and syndecan-2 are predominantly expressed in multiple myeloma, H9, and Jurkat cells, respectively, we could delineate the contribution of syndecan to cell death by downregulating a single syndecan in each cell line. We found that syndecan-1 knockdown in multiple myeloma cells greatly enhanced the sensitivity of cells to TRAIL-induced apoptosis (Fig. 2). We also observed that knockdown of syndecan-4 in H9 cells and downregulation of syndecan-2 in Jurkat cells led to an increased sensitivity to Fasl (Supplemental Fig. 3). Therefore, irrespective of the type of syndecan, removal of syndecan enhances TRAIL- and FasL-stimulated apoptosis. Our results illustrate that syndecan plays a negative role in the apoptosis initiated by TRAIL and FasL. We further found that increased TRAIL-mediated apoptosis in syndecan-1 knockdown cells was not due to changes in the expression of TRAIL receptors, c-FLIP, Mcl-1, Bel-2, Bim, or Bax (Fig. 4). Neither did a decrease in syndecan-1–associated heparan sulfate proteoglycan or integrin binding play a role in the observed increase in TRAIL-induced apoptosis (Supplemental Fig. 2). Furthermore, syndecan-1 knockdown did not affect the endocytosis of TRAIL receptors (Fig. 5). Instead, the enhanced TRAIL- and FasL-induced apoptosis in syndecan-1–deficient myeloma and lymphoma cells was associated with an increase in DISC formation and accelerated caspase-8 activation (Fig. 6, Supplemental Fig. 5). In addition, for TRAIL-induced apoptosis, the increased cell death in syndecan-1–deficient cells is partly attributed to an increase in O-glycosylation of TRAIL receptors, as a consequence of elevated GALNT3 expression.
Syndecan-1 is a target gene of Blimp-1. Blimp-1 knockdown has been shown to increase spontaneous cell death in H929 and U266 cells (31). We observed that cell death triggered by TNF-α, another DR ligand, was not affected by syndecan-1 knockdown in H929 cells (Fig. 2H). TRAIL and FasL are different from TNF-α in the process of DISC formation: whereas FADD is recruited by TRAIL and Fas to form DISC at the cell surface, FADD becomes associated with TRADD for the assembly of DISC in the cytosol after TRADD is dissociated from the membrane-bound TNFRI complex (17–19). The insensitivity of TNF-α–induced apoptosis to syndecan-1 knockdown suggests the possibility that syndecan affects the membrane-situated DR complex, but not with the cytoplasmic FADD-containing complex.

Syndecan-1 is a target gene of Blimp-1. Blimp-1 knockdown has been shown to increase spontaneous cell death in H929 and U266 cells (31). The fact that we observed elevated apoptosis in syndecan-1–deficient myeloma cells upon long-term culture (>2 mo) is consistent with an impaired growth of syndecan-1–deficient myeloma in vivo (11). However, we did not find any difference in cell viability within the few weeks during which these experiments were performed with each cell line. Thus, the increased DR-induced apoptosis in syndecan-1 knockdown cells observed in the current study was not due to elevated spontaneous cell death. The difference between Blimp-1 knockdown and syndecan-1 knockdown myeloma cells suggests that, in addition to syndecan-1, other Blimp-1 downstream genes also contribute to the survival of plasma cells and multiple myeloma. This is illustrated by the fact that Blimp-1 deficiency led to increased Bim and reduced Mcl-1 levels (31), whereas the levels of these two proteins were not altered by knockdown of syndecan-1 in myeloma cells (Fig. 4).

Syndecan is linked to the actin cytoskeleton through the C1 region of the intracellular domain (21). Fas is also associated to the actin filament through binding to ezrin/radixin/moesin (20, 38). We have recently demonstrated that knockdown of ezrin enhances the formation of DISC (25), suggesting that the linkage of Fas to ezrin-actin imposes a restriction that hampers the association of FADD and procaspase-8 with DRs to form DISC. The presence of syndecan at the cell membrane, through the association with cortical actin, may also exert constraints inhibiting the effective recruitment of FADD and procaspase-8. The removal of major syndecan most likely relieves these constraints and facilitates efficient assembly of DISC. In the current study, we also observed that TRAIL binding was accompanied with an immediate cleavage of the intracellular region of syndecan-1 (Fig. 1A, 1C, 1D), indicating that removal of the intracellular portion of syndecan-1 could be part of the processes for full assembly of DISC.

We have identified an additional mechanism that is TRAIL specific on the enhanced DISC formation in syndecan-1 knockdown cells. The expression of GALNT3, the enzyme that mediates the O-glycosylation of TRAIL receptor, was elevated in syndecan-1–downregulated myeloma cells (Fig. 7A). The sensitivity of cancer cells to TRAIL-triggered cell death has been found associated with the expression of GALNT isoforms (35). Levels of GALNT14 are correlated with sensitivity to TRAIL in pancreatic carcinoma, non–small-cell lung carcinoma, and malignant melanoma cell lines, whereas the expression of GALNT3 correlates with the susceptibility to TRAIL-induced apoptosis in colorectal cancer lines (35). Downregulation of O-glycosylation enzymes by siRNA confers resistance to TRAIL-triggered apoptosis in cancer cell lines. Overexpression of GALNT14 in TRAIL-resistant pancreatic cancer cell line leads to increased TRAIL-triggered cell death, accompanied with DISC formation and activation of caspase-8 and caspase-3 (35). As a consequence of increased O-glycosylation of TRAIL receptors, we observed increased DR4 clustering in syndecan-1 knockdown cells (Fig. 7B), similar to the enhanced translocation of DR4/DR4 oligomers into DISC complex previously reported for GALNT14 (35). It may be noted that we do not know how syndecan-1 downregulation promotes the expression of GALNT3. Syndecan-1 has been shown to transmit or modulate various signal pathways (5–7), and whether any of the signaling cascades is linked to GALNT expression is being determined.

TRAIL triggers apoptosis in multiple myeloma cell lines (27–29). TRAIL kills myeloma cells regardless of their resistance to chemotherapeutic drugs, and TRAIL is a potential therapeutic reagent for incurable multiple myeloma. However, many myeloma cells are only modestly sensitive to TRAIL, as also shown in the current study. Syndecan-1 is overexpressed in multiple myeloma cells, and contributes to the growth and metastasis of myeloma tumor (8, 11, 12). Syndecan-1 has thus become a target for treating myeloma, with approaches including specific knockdown of syndecan-1 (11) or anti–syndecan-1 immunoconjugate (39). Our results illustrate that, by downregulation of syndecan-1, myeloma cells become highly susceptible to TRAIL killing. Because de-
livery of syndecan-1–specific siRNA has been shown to knock down syndecan in vivo (11), the combinatorial therapy using syndecan-1 siRNA and TRAIL is now possible. Whether such combinatorial treatment will be effective against multiple myeloma deserves further investigation.

Among different DR agonists, TRAIL and TRAIL receptor agonists have displayed promising therapeutic effect toward solid tumors and lymphomas in series of Phase I and Phase II clinical trials (22–26). Notably, a few tumor cells are resistant to treatment with TRAIL alone. The resistance is often circumvented by combination of TRAIL with reagents such as cytotoxic drugs, proteasome inhibitors, or histone deacetylase inhibitors. Most of these reagents promote TRAIL-induced apoptosis by downregulating anti-apoptotic molecules (e.g., c-FLIP, Bcl-2), by upregulating proapoptotic proteins (e.g., Bak, Bak), or by increasing the expression of TRAIL-R1 and TRAIL-R2 (22–26). In the current study, we demonstrate that knockdown of syndecan-1 provides a distinct mechanism to sensitize tumor cell to TRAIL killing. Downregulation of syndecan-1 did not affect the expression of the aforementioned apoptosis-associated molecules and the surface levels of TRAIL receptors (Fig. 4). Instead, syndecan-1 knockdown promotes TRAIL receptor clustering and increased DISC formation. This suggests that syndecan downregulation could be used not only in promoting TRAIL-mediated apoptosis, but also in enhancing the efficacy of the current TRAIL combinatorial therapy. For cancer cells that predominantly display a specific type of syndecan, the feasibility of such an approach is being explored.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1. Expression of surface syndecan in H929, RPMI8226, and H9 cells. H929, RPMI8226 (A), and H9 cells (B) were stained with anti-syndecan-1, anti-syndecan-2, anti-syndecan-3, and anti-syndecan-4. The fluorescence intensity was analyzed by flow cytometry on a FACSCalibur system. Solid curve, both anti-syndecan and secondary antibody; shadowed curve, secondary antibody-only control.
Supplemental Figure 2. Removal of heparan sulfate or blockage of integrin binding does not enhance TRAIL-induced apoptosis. (A, C) Removal of heparan sulfate chains by heparinase III. H929 (A) and RPMI8226 (C) cells were treated without (Ctl) or with heparinase III (HepIII, 1 U/ml) for 1 h. Cell lysates were prepared, and the change in the molecular weight of syndecan-1 was determined by immunoblotting. (B, D) Heparinase III treatment reduced TRAIL-induced apoptosis. TRAIL was added to H929 (B) and RPMI8226 (D) cells pre-treated with or without heparinase III, and cell death was assessed by APC-Annexin V staining. (E, F) Blocking of syndecan-1-mediated integrin activation by synstatin (SSTN) peptide did not affect the sensitivity of myeloma cells to TRAIL-induced cell death. H929 (E) and RPMI 8226 (F) cells were treated with TRAIL in the presence or absence of SSTN peptide at the indicated doses for 6 h. Cell death was assessed by APC-Annexin V staining. Each data point represents triplicate determinations in a single experiment, and each experiment was repeated three times. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for paired t-test.
Supplemental Figure 3. Downregulation of the major syndecan enhances FasL-induced apoptosis in H9 and Jurkat cells. (A) Knockdown of syndecan-4 in H9 cells. Human T lymphoma H9 cells were transduced with pLL3.7 vector or with pLL3.7shSDC4, and GFP+ cells isolated by sorting. Surface syndecan-4 expression was determined by FACS. (B) Knockdown of syndecan-4 promotes FasL-induced apoptosis in H9 cells. Control and syndecan-4-knockdown H9 cells were treated with FasL at the indicated concentrations, and cell death was assessed 4 h later. (C) Accelerated caspase-8 and caspase-3 activation in syndecan-4-knockdown H9 cells after FasL treatment. Control and syndecan-4-knockdown H9 cells were treated with FasL (200 ng/ml), and total cell lysates were prepared at the indicated time points. The processing of procaspase-8 and procaspase-3 and the generation of active caspase-3 (caspase-3*) was determined by immunoblotting. (D) Downregulation of syndecan-2 in Jurkat cells. Jurkat cells were transduced with pLL3.7 vector or with pLL3.7shSDC2, and GFP-expressing cells sorted. The surface syndecan-2 expression was determined. (E) Knockdown of syndecan-2 in Jurkat cells enhanced FasL-induced apoptosis. Jurkat cells were treated with FasL at the indicated doses for 6 h, and cell death was determined. ***, P < 0.001 for paired t-test.
Supplemental Figure 4. TRAIL-induced apoptosis in H929 and U266 cells and the expression of Fas, c-FLIP, and FAP-1 in H9 cells are not affected by knockdown of syndecan-4. (A-D) Syndecan-4 downregulation in H929 and U266 cells did not affect TRAIL-mediated cell death. Control (pLL) and syndecan-4-knockdown (shSDC4) H929 (A) or U266 cells (B) were treated with TRAIL and cell death determined. Solid line, vector control; thin line, syndecan-4-knockdown; shadowed curve, secondary antibody-only control. (C, D) Normal levels of Fas and c-FLIP\(_L\) in syndecan-4-deficient H9 cells. The expression of Fas and c-FLIP\(_L\) in cell lysates from control and syndecan-4-knockdown H9 cells was determined by immunoblotting (C), and the surface Fas levels were determined by APC-conjugated anti-Fas staining and flow cytometry analysis (D). (E) FAP-1 was not altered in syndecan-4-knockdown H9 cells. The FAP-1 and active caspase-8 contents in FasL-treated control and syndecan-4-knockdown H9 cells were determined at the indicated time points by Western blots.