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Down-regulation of Fas-mediated apoptosis by plasma transglutaminase factor XIII that catalyzes fetal-specific cross-link of the Fas molecule

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ABSTRACT

The Fas antigen, also designated as APO-1 or CD95, is a member of the tumor necrosis factor receptor superfamily and can mediate apoptotic cell death in various cells. We report here that blood coagulation factor XIII (plasma transglutaminase, fibrin stabilizing factor) inhibits apoptosis induced by a cytotoxic anti-Fas monoclonal antibody in Jurkat cells. When cells were treated with the antibody in fetal calf serum-containing media, higher-molecular-weight (180 K) polypeptides containing Fas molecule were detected by immunoblotting. Under conditions where the transglutaminase activity was eliminated or suppressed, the cross-link of Fas was not observed, and concurrently cell death was hastened. Moreover, an antibody against factor XIII strongly accelerated the Fas-mediated apoptosis. Furthermore, addition of partially purified factor XIII neutralized the apoptosis-promoting effect of anti-factor XIII antibody, indicating that this enzyme is involved in cross-link of Fas and down-regulates Fas-mediated apoptotic cell death. Significantly, the cross-link of Fas was seen only in fetal calf serum but not in newly-born calf serum, 1-year-old calf serum or adult bovine serum. These data suggest that plasma transglutaminase factor XIII may play a key role in fetal development of vertebrates via cross-link of Fas antigen.

1. Introduction

During development and differentiation, a large number of cells should die by the mechanisms of programmed cell death. Apoptosis, a form of programmed cell death, is characterized by condensation of the cells, loss of plasma membrane microvilli, segmentation of the nucleus, formation of apoptotic body, and fragmentation of nucleosomal DNA [1,2]. Apoptosis is one of the most important mechanisms which regulates many cellular functions: embryogenesis, metamorphosis, clonal elimination of autoreactive B cells and T cells, and killing of virus-bearing or tumor cells by cytotoxic T cells, *etc.,* [3–5]. The signals of cell death can be mediated by various cell surface receptors, *e. g.*, B cell antigen receptor (surface immunoglobulin), T cell antigen receptor, and tumor necrosis receptor [3,4,6]. Regarding Fas antigen, a typical cell surface receptor protein that mediates apoptosis, mouse monoclonal antibody (mAb) which has a cytotoxic activity was first established using human diploid fibroblast FS-7 cells as an immunogen (anti-Fas antibody), and cDNA encoding human Fas antigen was isolated [7–9]. The Fas antigen belongs to the tumor necrosis factor (TNF) receptor superfamily, which includes the TNF receptors (p60 and p80), lymphotoxin β receptor, nerve growth factor receptor, TNF-related apoptosis-inducing ligand (TRAIL) receptors, CD27, CD30, CD40, CD134, *etc.,* [9–11]. The Fas antigen mediates apoptosis induced by binding with a Fas ligand (FasL) discovered on the cell surface of a cytotoxic T cell hybridoma [12]. FasL is a type II transmembrane protein belonging to TNF superfamily which contains TNFs (α and β), lymphotoxin β, nerve growth factor, TRAILs, the ligands for CD27, CD30, CD40 and CD134, *etc.,* [6,13,14]. Numerous studies on the Fas/FasL system using mutant mice and patients have revealed that this system plays an important role not only in immune cells but also in other organs, and catastrophe of this system causes various serious diseases such as immune diseases and cancer [6,15–17]. However, the regulation mechanisms of Fas-mediated apoptosis remain poorly understand even now.

On the other hand, tissue transglutaminase, one of transglutaminases (EC 2. 3. 2. 13; R-glutamyl peptide: amine-γ-glutamyltransferase) that catalyze the formation of γ-glutamyl-ε-lysine bridge between several proteins is known as a modulator in apoptosis [18,19]. Tissue transglutaminase is induced and activated in

Abbreviations: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester; FasL, Fas ligand; FCS, fetal calf serum; KLH, keyhole lympet hemocyanin; mAb, monoclonal antibody; MDC, monodansylcadaverine; SAF, serum anti-apoptosis factor; TCA, trichloroacetic acid; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

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cells undergoing apoptosis. The activation of tissue transglutaminase in apoptotic cells results in the accumulation of cross-linked intracellular proteins. In this paper, we demonstrate that plasma transglutaminase, but not tissue transglutaminase, inhibits apoptosis by catalyzing cross-link of Fas molecule. Plasma transglutaminase, the blood coagulation factor XIII is well-characterized to catalyze cross-linking of fibrin in the presence of calcium ions in blood clotting [20,21]. We propose here novel role of plasma transglutaminase factor XIII relating to the regulation of Fas-mediated apoptosis.

2. Materials and methods

2.1. Materials

Bovine serum albumin, EGTA, insulin–transferrin–sodium selenite media supplement, keyhole lympet hemocyanin (KLH), L-lysine, monodansylcadaverine (MDC), proteinase K, RNase A were purchased from Sigma (St. Louis, MO, USA). Polyvinylidene difluoride membrane was from Millipore (Billerica, MA, USA). RPMI1640 culture medium was from Nissui (Tokyo, Japan). Sera were from IBL (Gunma, Japan). Trypan blue was from Merck (Darmstadt, Germany). Alkaline phosphatase-conjugated anti-rabbit IgG was from Promega (Fitchburg, WI, USA). Anti-human Fas mAbs were from MBL (Nagoya, Japan). Rabbit anti-coagulation factor XIII a-subunit antibody and human placenta factor XIII a subunit were from Calbiochem (San Diego, CA, USA).

2.2. Peptide synthesis and antibodies preparation

Peptides were synthesized on a model 430A solid phase peptide synthesizer (Applied Biosystems, Foster City, CA, USA), deprotected, and purified by high performance liquid chromatography. Two peptides were synthesized and used for immunogens: NH_2-CITSDSENSNFRNEIQSLV-COOH (Fas C) corresponding to the carboxyl terminal region (res. 302–319); and NH_2-CRKHRKENQG-SHESPTLNP-COOH (Fas 183) corresponding to residues 183–201 of human Fas antigen. These peptides were conjugated with KLH, and immunized rabbits with the conjugates as described previously [22–24]. Purification and characterization of the antibodies were performed as described previously [22–24].

2.3. Cell culture

Human Jurkat T cells, provided by Japan Cancer Research Resources Bank, were grown in RPMI1640 culture medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin G and 200 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 [25].

2.4. Immunoblotting

Cells were collected by centrifugation and washed with phosphate-buffered saline containing 5 mM EDTA, and treated with 10% trichloroacetic acid (TCA) for 10 min at 0 °C. TCA-treated cells were dissolved by sonication in 50 μl of solubilization solution (7.2 M urea, 1.6% Triton X-100, 4 mM 2-mercaptoethanol, 40 mM Tris–HCl (pH 6.8), 3% lithium dodecylsulfate and 8% glycerol). The samples were subjected to SDS–PAGE followed by immunoblotting as described previously [22–24].

2.5. Other methods

Cell viability assay was performed by the trypan blue dye exclusion method [3,24–26]. Transglutaminase activity was determined by the fluorometric activity of the incorporation of MDC into casein as described previously [27].

3. Results and discussion

3.1. Cross-link of Fas antigen during apoptosis induced by anti-Fas mAb

Jurkat cells express a 45-kDa Fas antigen as judged by staining with an anti-peptide antibody anti-Fas C against its carboxyl-terminal region (Fig. 1A, lanes 1 and 2). This antibody can be absorbed with the specific synthetic peptide Fas C that was used as an immunogen (Fig. 1C). When cells were incubated with an anti-Fas mAb cytotoxic to them, a 180-kDa polypeptide cross-reacting with the anti-peptide antibody appeared in an hour (Fig. 1A, lanes 3–5). Longer incubation resulted in appearance of other higher-molecular-weight Fas-related polypeptides as well as in a decrease in the 45-kDa native Fas (Fig. 1A). A similar immunoblotting profile was obtained with another anti-peptide antibody, anti-Fas 183, against residues 183–201 of human Fas (Fig. 1B). Although these results showed that the 180-kDa polypeptide obviously contains Fas molecule(s), we do not know whether the 180-kDa polypeptide contains any other molecule(s) or not. The 180-kDa polypeptide did not contain the mAb used for apoptotic induction, since it was not stained with an antibody against mouse IgM (Fig. 1D). However, further studies are needed to characterize the 180-kDa polypeptide containing Fas molecule.

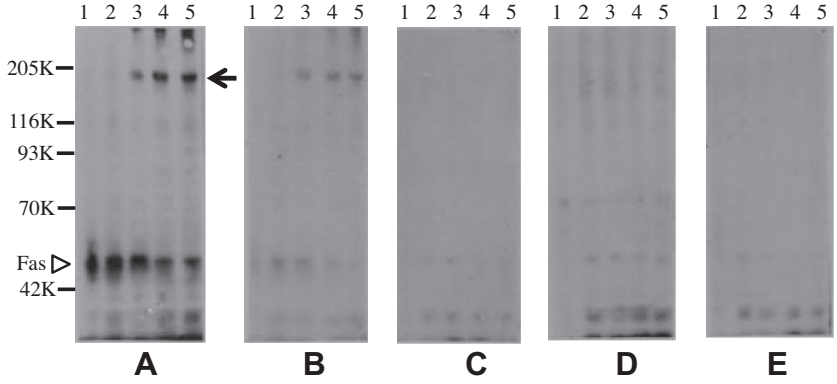

Fig. 1. Cross-link of the Fas antigen in Jurkat cells after stimulation with anti-Fas mAb. The cells were treated with anti-Fas mAb (final concentration, 50 ng/ml) for 0 h (lane 2), 1 h (lane 3), 3 h (lane 4), and 6 h (lane 5), lysed, and subjected to immunoblotting (3 μl aliquates corresponding to 9×10^4 cells per lane) using the anti-Fas C antibody. In lane 1 of each panel, membrane fraction of Jurkat cells was electrophoresed. The 180-kDa band stained with anti-Fas C is arrowed. The native Fas is indicated by an open triangle. Antibodies and conditions were: (A) anti-Fas C; (B) anti-Fas 183; (C) anti-Fas C with a carboxyl-terminal peptide (Fas C); (D) anti-mouse IgM; and (E) normal rabbit IgG. Apparent molecular weights of marker proteins are indicated.

3.2. Effects of various treatments on the cross-link of the Fas antigen

To determine the factors participating in the cross-link of Fas, we depleted serum from the medium to which growth factors were supplemented instead. The cells were viable for at least 3 days under such conditions. The cross-link of Fas was not observed when Fas-mediated apoptosis was initiated in a serum-free medium (Fig. 2A, lane 3), indicating that the reaction involves serum factors. Serum contains transglutaminases known as blood coagulation factor XIII. The cross-link of Fas was suppressed by incubation of the cells with the anti-Fas mAb in the presence of L-lysine, MDC or EGTA, which inhibit the activity of transglutaminase (Fig. 2, lanes 4–6). The 180-kDa band did not appear even after longer incubation (data not shown). On the other hand, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), a chelator of intracellular calcium, did not affect the cross-link (Fig. 2, lane 7). These findings suggest that plasma transglutaminases catalyze the cross-linking of Fas, which is induced by binding with the cytotoxic anti-Fas mAb. This was later confirmed by the results of the experiment using an antibody against the factor XIII (see Fig. 3).

3.3. Effects of various treatments on apoptotic cell death mediated by Fas

Next, the role of plasma transglutaminase factor XIII in Fas-mediated apoptotic cell death was examined using cell viability assays. Cell viability was retained for 24 h when serum was removed from the medium or 10 mM L-lysine was supplemented. By further addition of the cytotoxic mAb, 80% of the cells became stained with trypan blue, while 75% of the cells were viable 24 h after treatment with the antibody under the control conditions (Fig. 2B). The enhancement of cell death correlated well with the suppression of cross-linking of Fas induced by the mAb (see Fig. 2A).

3.4. Effects of an anti-factor XIII antibody on cross-link of the Fas antigen and apoptosis of Jurkat cells

We carried out a series of experiments using an antibody against factor XIII to prove that plasma transglutaminase suppresses Fas-mediated apoptosis by cross-linking the Fas molecule. This antibody certainly inhibited the activity of factor XIII (Fig. 3A). As shown in Fig. 3B, pretreatment of the culture medium with increasing concentrations of anti-factor XIII antibody depressed the intensity of the180-kDa band in a dose-dependent manner, clearly demonstrating that cross-linking of Fas in apoptotic cells is catalyzed by plasma transglutaminase, factor XIII itself or its related enzyme. In addition, population of trypan blue-sensitive cells were also enhanced by the addition of the anti-factor XIII antibody to the incubation media (Fig. 3C). IgG from unimmunized rabbits did not influence the characteristics of the cells in these experiments (data not shown). Moreover, cell viability was moderately recovered by the addition of a partially purified human factor XIII a subunit (Fig. 3D).

3.5. Concluding remarks

Eight types of transglutaminases have been established: blood coagulation factor XIII (plasma transglutaminase), keratinocyte transglutaminases, tissue transglutaminases, epidermal transglutaminase, prostate transglutaminases and transglutaminases X–Z [28]. Among them, tissue transglutaminases that are accumulated in some apoptotic cells are thought to be involved in apoptotic cell death, which is presumably an intracellular event [18,19]. However, in this study, the enzyme of interest exists in the serum used for culture media. Although we do not know whether the antibody against factor XIII inhibits the activity of tissue transglutaminases, the antibody cannot probably recognize tissue transglutaminases because of the low homology between factor XIII and them. In addition, it is unlikely here that intracellular enzymes participate in the cross-link of Fas, since neither of inhibitors nor neutralizing antibody used here can affect the cytosolic transglutaminases.

As is well known, Fas mediates apoptotic cell death induced by binding with a FasL *in vivo* [12]. Although we also carried out induction of Fas-mediated apoptosis using soluble FasL instead of cytotoxic anti-Fas antibody, unfortunately, the 180-kDa polypeptide generated by polymerization of Fas could not be detected (data not shown). The much higher (rather than soluble FasL used) cytotoxic activity of the anti-Fas mAb probably resulted in remarkable generation of the 180-kDa polypeptide. Potentially, the

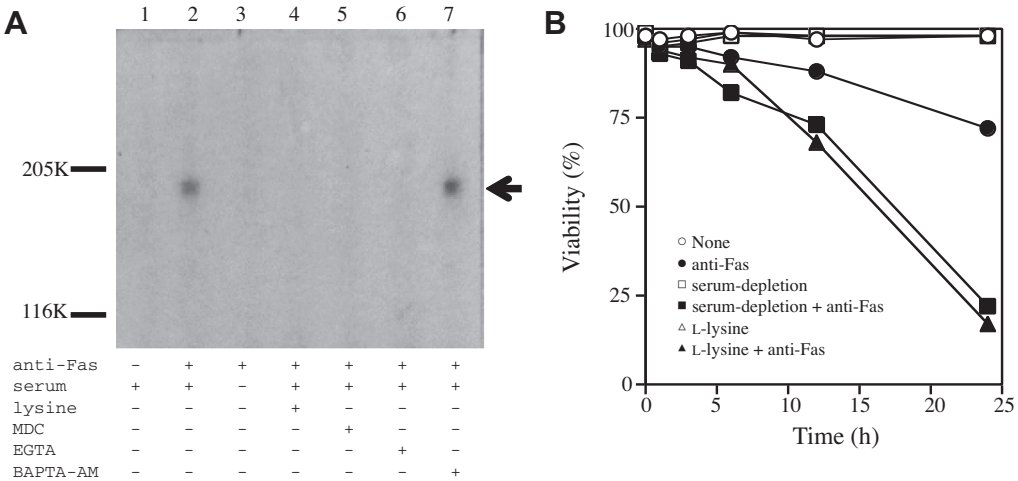

Fig. 2. Effects of various treatments on the cross-link of the Fas antigen and apoptotic cell death mediated by Fas antigen. (A) Cross-link of the Fas antigen. Jurkat cells (1.5×10^6 cells) resuspended in 5 ml of medium were treated with the anti-Fas mAb (final concentration, 50 ng/ml) under various conditions: serum-depletion, with 10 mM L-lysine, with 625 μM MDC, with 5 mM EGTA, with 25 μM BAPTA-AM for 1 h. Lane 1 shows Jurkat cells without antibody. Samples loaded to SDS–PAGE were equivalent to 3×10^4 cells. The 180-kDa band stained with anti-Fas C is arrowed. The apparent molecular weights of marker proteins are indicated. (B) Cell viability. Jurkat cells were incubated in medium under various conditions; none (open circle), 50 ng/ml anti-Fas mAb (closed circle), serum-depletion (open square), serum-depletion and 50 ng/ml anti-Fas mAb (closed square), 10 mM L-lysine (open triangle), and 10 mM L-lysine plus 50 ng/ml anti-Fas mAb (closed triangle). Viable cells were counted by the trypan blue dye exclusion method.

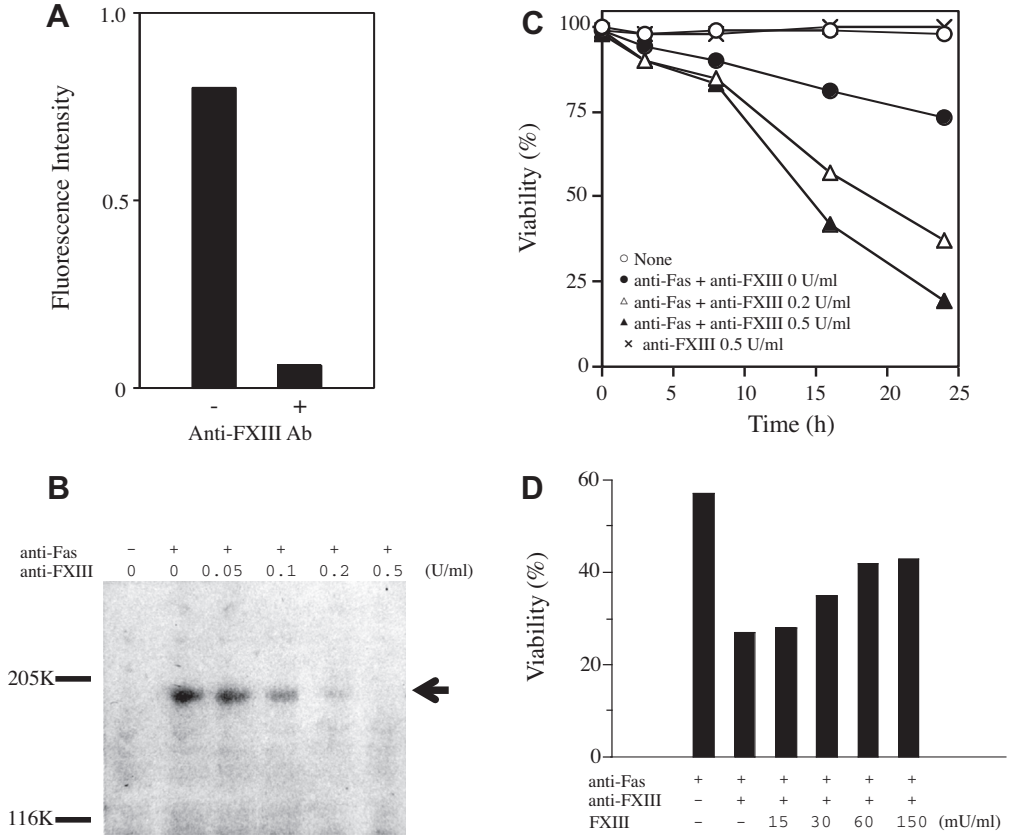

Fig. 3. Effects of an anti-coagulation factor XIII antibody on cross-link of the Fas antigen and apoptosis of Jurkat cells. (A) Effects of an anti-coagulation factor XIII antibody on transglutaminase activity. A preparation corresponding to 3×10^{-3} U/ml human placenta factor XIII a subunit was incubated with or without 0.5 U/ml anti-factor XIII antibody for 30 min at 30 °C. Transglutaminase activity was determined as described in "Section 2". (B) Inhibition of cross-link of the Fas antigen by an anti-coagulation factor XIII antibody. Jurkat cells (5×10^5 cells) were suspended in 0.5 ml of serum-containing medium pre-incubated with an anti-coagulation factor XIII a-subunit antibody to concentrations of 0 U/ml, 0.05 U/ml, 0.1 U/ml, 0.2 U/ml and 0.5 U/ml for 30 min at 37 °C, and incubated without or with 50 ng/ml anti-Fas mAb for 1 h at 37 °C. The 180-kDa band stained with anti-Fas C is arrowed. The apparent molecular weights of marker proteins are indicated. (C) Acceleration of the Fas-mediated apoptotic cell death by the anti-factor XIII antibody. Jurkat cells, resuspended in the medium treated with the anti-factor XIII antibody to concentrations of 0 U/ml (circles), 0.2 U/ml (open triangle) and 0.5 U/ml (closed triangle and cross) were cultured without (open circle and cross) or with (closed circle and triangles) 50 ng/ml anti-Fas mAb for 0–24 h at 37 °C, and viability of the cells was examined. (D) Recovery of the Fas-mediated apoptosis by addition of coagulation factor XIII. Serum-containing medium was preincubated with thrombin-activated coagulation factor XIII to concentrations of 0 mU/ml, 15 mU/ml, 30 mU/ml, 60 mU/ml, and 150 mU/ml for 30 min at 37 °C, and then treated without or with 0.2 U/ml anti-coagulation factor XIII antibody for 30 min at 37 °C. Jurkat cells (1×10^6 cells) were suspended in 1 ml of the medium, and incubated with anti-Fas mAb (final concentration, 50 ng/ml) for 24 h at 37 °C. Viable cells were counted as described in "Section 2".

possibility that factor XIII-mediated cross-link of cell surface receptors including Fas can protect cells from self-reactive antibodies that mimic activities of their ligands could not be eliminated. These problems should be elucidated in the future.

This system, suppressing Fas-mediated apoptosis of Jurkat cells, must involve not only plasma transglutaminase factor XIII but also other fetal-specific factor(s) in serum, since cross-link of Fas was seen only in FCS but not in newly-born calf serum, 1-year-old calf serum or adult bovine serum (Fig. 4). It is noteworthy that we have demonstrated in FCS a novel apoptosis-suppressing system including serum anti-apoptosis factor (SAF) that is specific for FCS but not found in newly-born, 1-year-old calf or adult bovine serum [25]. Data obtained in this study, together with our previous report [25], suggest that suppression of Fas-mediated apoptosis by serum factors is catalyzed by extracellular transglutaminase (probably factor XIII), the activity of which is regulated by SAF, a possible key factor for apoptosis in the development and homeostasis of the immune system.

Anyhow, this is the first report that plasma transglutaminase factor XIII targets an apoptotic molecule to down-regulate its signals when cell death is initiated by an agonist. Our results revealed a new inherent function of factor XIII; this enzyme may play a key role (probably collaborate with SAF) in the stages of prenatal

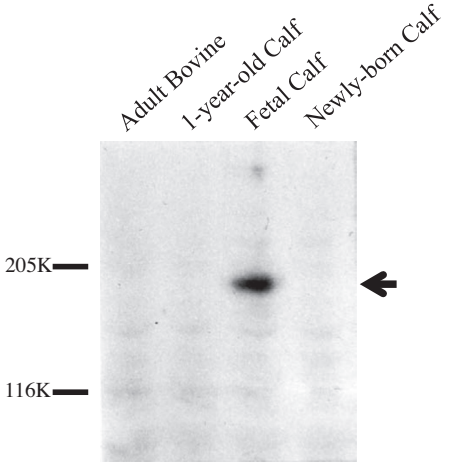

Fig. 4. Effects of origin of sera on cross-link of Fas. Jurkat cells were incubated with anti-Fas mAb (final concentration, 50 ng/ml) in RPMI1640 medium containing 10% adult bovine serum, 1-year-old calf serum, fetal calf serum or newly-born calf serum for 1 h at 37 °C. The treated cells were lysed and subjected to immunoblotting (3 μl aliquates corresponding to 9 × 104 cells per lane) using the anti-Fas C antibody. The 180-kDa band stained with anti-Fas C is arrowed. Apparent molecular weights of marker proteins are indicated.

development via cross-link of Fas antigen followed by apoptosis suppression. Further studies will be needed to clarify the characterization of SAF, physiological role and the molecular mechanism of down-regulation of apoptosis by factor XIII (and also SAF)-mediated cross-link of Fas.

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