Published in final edited form as:

Exp Cell Res. 2013 May 1; 319(8): 1061–1072. doi:10.1016/j.yexcr.2013.02.020.

CEACAM1 Regulates Fas-mediated Apoptosis in Jurkat T-cells via its Interaction with β -catenin

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Abstract

CEACAM1 (Carcinoembryonic Antigen Cell Adhesion molecule 1), an activation induced cell surface marker of T-cells, modulates the T-cell immune response by inhibition of the T-cell and IL-2 receptors. Since T-cells undergo activation induced cell death via Fas activation, it was of interest to determine if this pathway was also affected by CEACAM1. Previously, we identified a novel biochemical interaction between CEACAM1 and the armadillo repeats of β -catenin in Jurkat cells, in which two critical residues, H469 and K470 of the cytoplasmic domain of CEACAM1-4L played an essential role; while in other studies, β -catenin was shown to regulate Fas-mediated apoptosis in Jurkat cells. CEACAM1 expression in Jurkat cells leads to the re-distribution of β -catenin to the actin cytoskeleton as well as inhibition of β -catenin tyrosine phosphorylation and its degradation after Fas stimulation. As a result, Fas-mediated apoptosis in these cells was inhibited. The K470A mutation of CEACAM1 partially rescued the inhibitory effect, in agreement with the prediction that a CEACAM1- β -catenin interaction pathway is involved. Although CEACAM1 has two ITIMs, they were not tyrosine-phosphorylated upon Fas ligation, indicating an ITIM independent mechanism; however, mutation of the critical residue S508, located between the ITIMs, to aspartic acid and a prerequisite for ITIM activation, abrogates the inhibitory activity of CEACAM1 to Fas-mediated apoptosis. Since Fas-mediated apoptosis is a major form of activation-induced cell death, our finding supports the idea that CEACAM1 is a general inhibitory molecule for T-cell activation utilizing a variety of pathways.

Keywords

CEACAM1; Carcinoembryonic antigen-related cell adhesion molecule-1; apoptosis; β -catenin; Fas; T-cell; Jurkat cell; actin cytoskeleton

Introduction

CEACAM1 is a transmembrane cell adhesion molecule that belongs to the CEA superfamily. There are more than ten splicing isoforms of CEACAM1, with either a long or a short cytoplasmic domain and 1-4 Ig-like extracellular domains. CEACAM1 is expressed

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There are no conflicts of interest.

in various tissues including epithelial, endothelial and hematopoietic cells. Unlike in most tissues where both long and short isoforms are expressed and the short isoform is the major regulatory molecule in epithelial cells [1], the long cytoplasmic isoforms of CEACAM1 (e.g., CEACAM1-4L) but not the short isoform, is predominantly expressed in activated human T-cells as a co-inhibitory molecule [2]. Previous studies have established that recruitment of SHP-1 by phosphorylated ITIMs in the cytoplasmic domain of CEACAM1-4L inhibit T-cell proliferation and functions via inhibition of both IL-2 [3] and TCR [4] signaling resulting in the down-modulation of the immune response.

More recently, we have shown that a second conserved inhibitory motif that binds the Arm repeats of β -catenin is also found in the cytoplasmic domain of CEACAM1-4L [5]. We showed that CEACAM1-4L co-localized with β -catenin in membranous specks in Jurkat cells and that mutation of two key residues (H469A and K470A) within the Arm-binding motif substantially reduced β -catenin binding in GST-pull down assays.

The implications are provocative since β -catenin is thought to play a critical role in T-cell development and survival [6-8], and deregulation of the β -catenin pathway is involved in development of hematopoietic malignancies such as leukemia [6, 9-10]. In addition, stabilized β -catenin potentiates Fas-mediated apoptosis in T-cells in a transgenic mouse model, and activated T-cells are highly proliferative and undergo activation induced cell death, mainly through Fas-mediated apoptosis [11]. Nonetheless, the functional significance of the Arm-binding motif in CEACAM1 is unknown. Since CEACAM1 also regulates apoptosis in several models including mammary morphogenesis [1], CD19 induced B-cell apoptosis [12] and spontaneous apoptosis in monocytes [13], and is down-regulated in leukemia patients [14], we investigated the possibility that the CEACAM1- β -catenin interaction might also regulate Fas-mediated apoptosis in T-cells as a way to fine-tune the T-cell response.

Jurkat cells are human T-cell leukemia cells which are extremely susceptible to apoptotic stimuli, including Fas ligation. They are widely used in apoptosis studies especially in activation induced cell death [10-11, 15]. Jurkat cells also have elevated β -catenin expression compared to normal T-cells [10] but CEACAM1 expression is absent [5]; thus Jurkat cells serve as a good model for our study of CEACAM1- β -catenin involvement during T-cell apoptosis.

Material and Methods

Cell culture and reagents

Jurkat cells were obtained from ATCC. Stable transfection of CEACAM1-4L and 4S wild type were described before [5] and cells with CEACAM1-4L mutants were obtained similarly. Cells were cultured in RPMI 1640 media (Mediatech) supplemented with 10% FBS (Omega Scientific) and 1% antibiotics-anti-mycotics (Mediatech) [5]. PBMCs were isolated and maintained as described [5].

Antibodies

CEACAM1 antibodies, T84.1, 5F4 and long-form specific 22-9 were previously described [5]. PE-anti-CEACAM1 antibody was obtained from R&D systems. antibodies to β -catenin, SHP1 and FITC-conjugated anti-CD95 antibody were purchased from BD Biosciences. Antibodies to β -actin, Fas and FasL were from Santa Cruz, and Caspase 3 and Caspase 8 antibodies from Calbiochem. Phospho-tyrosine specific antibody 4G10 and Fas-activating antibody CH11 were from Upstate. Secondary antibodies for western blotting, goat anti rabbit 800 nm and goat anti mouse 680 nm were from LiCor.

Other reagents

Inhibitors for Caspase 3 (Z-DEVD-FMK), 8 (Z-IETD-FMK), Pan-Caspase (Z-VAD-FMK); negative control for Caspase inhibitors (Z-FA-FMK), Caspase 8-FITC substrate (FITC-IETD-FMK), camptothecin and calpain inhibitors ALLN (Ac-LLnL-CHO) and calpeptin were all obtained from Calbiochem. NucView 488 Caspase 3 substrate was obtained from Biotium. Protein-A/G plus agarose beads for immunoprecipitation were purchased from Pierce Protein Biology.

Site-Directed Mutagenesis

H469A, K470A, HK2A (the double mutation of H469 and K470 to Alanines, H469A/K470A), S508A, S508D mutated forms of CEACAM1-4L in the pH β -actin vector [1] were obtained using Quikchange XL (Stratagene) per company protocol. Primers were designed by Quikchange primer design using NCBI Reference Sequence NM_001712.4.

Apoptosis Assay

Cells were cultured at 4×10^5 /mL and treated with anti-Fas antibody CH11 (Upstate) or other reagents for the indicated time and dose. After treatment, cells were collected, washed with PBS and Annexin V Binding buffer (BD Biosciences), suspended in Annexin V binding buffer and stained with APC-Annexin V and PI (BD Biosciences). Data were acquired by FACS Canto II and analyzed by FlowJo.

Western blot

Cells were washed twice with PBS, lysed by NP-40 lysis buffer (10 mM Tris, 100 mM NaCl, 10% glycerol, 1 mM EDTA, EGTA, PMSF, Na₃VO₄, 1% NP-40, 0.5mM sodium deoxycholate, proteinase inhibitor cocktail (Roche) and halt-phosphatase inhibitor cocktail (Pierce Protein Biology)) on ice for 30 min and centrifuged at 13000 rpm, 4 °C for 30 min. For phosphorylation studies, pervanadate was prepared freshly as described [5]. Cells were suspended in 10 mL PBS and treated with 2 mM freshly made pervanadate for 15 min before harvest. Protein concentration was determined by Bradford Assay (Bio-Rad). Fifty micrograms of extracted proteins from each sample were separated on NuPAGE Bis-Tris gel (Invitrogen), transferred to fl-PVDF membranes (Millipore), blocked with blocking buffer (Licor) and probed with appropriate antibodies then scanned and analyzed by Odyssey Imaging System (Licor).

Immunoprecipitation

Immunoprecipitation experiments were performed using cell lysates as described above. For -catenin-actin co-IPs, Triton-X 100 was used in place of NP-40 [10]. One to four milligram of cell lysates were precleared with protein-A/G plus beads (Pierce Protein Biology) coupled with mouse IgG, incubated with 2-5 ug antibodies overnight with gentle rocking at 4 °C. Protein A/G plus beads were added to pull down antibody-protein complex and incubated for additional 4 hrs. Unbound proteins were washed off with PBS or lysis buffer for three times. Proteins were eluted by 2x LDS sample buffer (Invitrogen) at 95°C for 5 min and resolved by western blot analysis.

Flow Cytometry

For expression of CEACAM1 and Fas, cells were incubated with one ug/mL CD66a-PE (R&D Systems) and/or Fas-FITC (BD) for 15 min at 4 °C. Fluorescence intensity was measured on a FACS Canto II (BD Biosciences) and analyzed by Flowjo Flow Cytometry Analysis Software (Tree Star). Caspase 3 and 8 activities were measured by NucView 488 Caspase 3 substrate (Biotium) or Caspase 8-FITC substrate (Calbiochem), respectively.

Cytoskeleton Staining

Jurkat cells with or without anti-Fas treatment were stained for cytoskeleton associated β -catenin as previously described [10, 16] using FITC labeled β -catenin antibody (BD biosciences). Briefly, cells were attached to poly-lysine coated glass slides overnight, washed with cytoskeleton buffer (CSK; 10 mM PIPES, 300mM Sucrose, 50 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100, protease inhibitor (Pierce Protein Biology), pH7.0) for 20 min at 4 °C, rinsed with PBS, fixed with 4% paraformaldehyde and followed by immunofluorescent staining [5]. Pictures were taken by Zeiss Microscope and analyzed using LSM 510 software [5].

Results

Expression of CEACAM1-4L and H469A/K470A mutants in Jurkat cells

Although Jurkat cells express β -catenin which has been shown to be involved in Fas mediated apoptosis [10], they do not express CEACAM1 which is an inhibitory marker of activated T-cells [5]. Since CEACAM1 has a β -catenin binding site in its cytoplasmic domain (Figure 1) in which two key residues were identified [5], we were interested in determining if CEACAM1 or its mutated forms would affect Fas-mediated apoptosis. When Jurkat cells were stably transfected with wild type, H469A or K470A mutants they had equivalent expression of CEACAM1-4L by FACS (Figure 2) and western blot analysis (data not shown). Since β -catenin was shown to regulate T-cell apoptosis by promoting Fas expression in mice [17], we first tested whether CEACAM1-4L expression in Jurkat cells inhibits β -catenin target gene expression by up-regulation of Fas. As shown in Figure 2, there were no apparent changes in the level of Fas expression on the cell surface after CEACAM1-4L wild type or mutant transfection. Since two canonical β -catenin target genes, cyclin D1 and c-myc, are not affected after CEACAM1-4L expression (data not shown), this suggests that CEACAM1-4L expression does not affect Fas expression or the regulation of β -catenin nuclear signaling in Jurkat cells.

CEACAM1-4L inhibits Fas induced apoptosis

The anti-Fas antibody CH11 that induces Fas activation and apoptosis in Jurkat cells [10], induced about 45% cellular apoptosis in untransfected cells after four hours treatment (25 ng/mL) as shown by Annexin V staining (Figure 3A). Under the same conditions, only 20% of Jurkat cells expressing CEACAM1-4L were apoptotic. At a higher dosage of 100 ng/mL, control cells are around 40% and 80% apoptotic after two or four hours of treatment, respectively, whereas the CEACAM1-4L expressing cells are about 20% and 50% apoptotic at the same time and dose. Thus, CEACAM1-4L rendered these cells about two fold less sensitive to Fas-induced apoptosis at various times or dose.

An equivalent inhibition of apoptosis was found for cells expressing the H469A mutant compared to wild type CEACAM1-4L, while the K470A significantly restored the level of apoptosis in these cells, exhibiting ~50% Annexin V positive cells at the dose of 25 ng/mL and treatment time of four hours (Figure 3A). When Jurkat cells expressing CEACAM1-4L containing the double mutation of H469A and K470A (CEACAM1-HK2A) were tested, the results were similar to the single mutant of K470A (Figure S1), demonstrating that K470 is a critical residue for its inhibitory activity.

Taken together these results demonstrate that CEACAM1-4L protects Jurkat cells from Fas mediated apoptosis and that K470 (but not H469) plays a critical role. Since the K470A mutant has been shown to inhibit the interaction of CEACAM1-4L with β -catenin in vitro and in GST pull-down assays with Jurkat cell lysates [5] and β -catenin plays a role in Fas-

mediated apoptosis [10], it is likely that CEACAM1-4L and β -catenin interact in the same Fas-mediated pathway.

Since CEACAM1 is a cell-cell adhesion molecule, Jurkat cells were also transfected with CEACAM1-4S, an isoform with the same extracellular domains but a short cytoplasmic domain which lacks both the ITIMs and the Arm-binding motif found in the cytoplasmic domain of the long isoform (Figure 1). When treated with anti-Fas the CEACAM1-4S transfected cells gave the same degree of inhibition of apoptosis as CEACAM1-4L transfected cells, suggesting that the cell-cell binding activity of CEACAM1 plays an important role and that the short isoform may transduce a protective signal, albeit via a different mechanism. In one respect the finding for the short isoform is surprising since it has been shown to mediate apoptosis in breast epithelial cells undergoing lumen formation [1]. The short cytoplasmic domain isoform interacts with the actin cytoskeleton and regulates apoptosis via calpain-9 in breast epithelial cells [1, 18]. Therefore, we tested whether calpain pathways were involved in these CEACAM1 expressing cells. However, unlike epithelial cells in which calpain inhibitors inhibit apoptosis [18], apoptosis is stimulated in Jurkat cells by calpain inhibitors, including peptide ALLN (Ac-LLnL-CHO) or calpeptin [19]. In our study there was no change in the percentage of apoptosis for cells with or without CEACAM1 expression treated with either calpain inhibitors (Figure S2), suggesting that both CEACAM1 isoforms utilize a calpain-independent pathway during Fas-mediated apoptosis in Jurkat cells. A possible mechanism of action involving the actin cytoskeleton for the short form, which is not expressed in activated T-cells, is discussed later.

Programmed cell death may proceed by either extrinsic (e.g., Fas-mediated) or intrinsic pathways (mitochondrial or DNA damage). As a control, the intrinsic pathway was also tested by camptothecin (CPT), a compound known to induce apoptosis via DNA damage [20]. This is an important point since the short isoform operates via the intrinsic pathway in the breast epithelial cell model [18]. Treatment dose and time were adjusted to produce 50-60% apoptosis in control cells at 10 μ M for 4 hours. Neither the wild type CEACAM1-4L nor the H469A mutation affected CPT induced apoptosis, indicating that CEACAM1-4L is only protective in the extrinsic Fas-mediated pathway (Figure 3A). However, the K470A mutant inhibited apoptosis after CPT treatment compared to control cells, suggesting a second mechanism exists that regulates programmed cell death via intrinsic pathway, but only when the Arm-binding motif is disrupted. These results indicate the possibility that both the extrinsic and intrinsic pathways are affected by the interaction of CEACAM1-4L with β -catenin.

In addition to Annexin V staining which measures an early stage of apoptosis, we also evaluated the effect of CEACAM1 on Fas-induced cell death by PI staining which measures late stage apoptosis and/or necrosis. Cell death was minimal with no difference in PI staining for controls and CEACAM1 expressing cells over the 4 hours treatment time period at all doses of anti-Fas antibody, suggesting an inhibitory effect specific to the early stage of apoptosis (Figure 3B). After twenty-four hours of treatment, all cells were at a late stage of apoptosis showing over 90% of Annexin V and PI double positive staining with no significant differences among the various groups. This suggests that CEACAM1 inhibits Fas-stimulated apoptosis in Jurkat cells in delaying but not completely blocking programmed cell death.

Inhibition of Caspase 3/8 activity by CEACAM1 expression

After Fas activation, the FADD complex activates Caspase 8, which after a downstream network of reactions converts pro-Caspase-3 to effector active Caspase-3 in the apoptosome [21]. Since CEACAM1 expression inhibited Fas mediated cell apoptosis, we examined

activation of Caspase 8 and Caspase 3 after anti-Fas stimulation at 25 ng/mL for 4 hours, the dosage that results in about 50% apoptosis (Figure 4A). Caspase activities were tested by binding to fluorescent labeled Caspase 3 and 8 substrates. As shown in Figure 4B and C, both Caspase 3 and 8 activities were reduced by CEACAM1-4L expression. In wild type CEACAM1-4L expressing cells, the levels of cleaved Caspase 3 and 8 are both reduced compared to non-CEACAM1 expressing cells after stimulation and CEACAM1-4L expressing cells maintained a higher level of pro-Caspase 3/8 as shown by western blotting (Figure 4D). This suggests that the mechanism by which CEACAM1-4L modulates the cellular response to Fas signaling involves the regulation of Caspase 3/8 activation, and is probably upstream of Caspase 8 as suggested by the canonical Fas-FADD-Caspase 8-Caspase 3 apoptotic pathway [21]. In addition, treatment with Caspase inhibitors including pan-, 3 and 8, but not control inhibitors, inhibited Fas-mediated apoptosis in both CEACAM1-4L expressing and control cells completely (Figure S3) indicating that CEACAM1 regulates Fas-mediated at the early stage of apoptosis (Figure 3A) and not necrotic cell death (Figure 3B).

Fas mediated proteolysis and phosphorylation of β -catenin

Chung et al. [10] showed that β -catenin translocates to the cytoskeleton and is degraded in the proteasome after treatment of Jurkat cells with anti-Fas antibody. From this finding they conclude that β -catenin plays a critical role in leukemic cell survival. Since we had already shown that CEACAM1-4L protected these cells from Caspase-dependent, Fas-mediated apoptosis, it was important to determine if CEACAM1-4L also protected β -catenin from anti-Fas induced degradation. As expected, Jurkat cells treated with anti-Fas antibody exhibited significant cleavage of β -catenin as evidenced by a lower molecular size instead of a full-length 92kDa β -catenin band, ~90kDa, in lower intensity (Figure 5A). In contrast, β -catenin in Jurkat cells expressing CEACAM1-4L exhibited mainly intact 92kDa β -catenin, a band not observed in control treated cells, as well as the degraded 90kDa band with higher intensity than in control treated cells (Figure 5A). Similarly, when β -catenin was immunoprecipitated, a procedure that increased the β -catenin intensity after immunoblotting with anti- β -catenin antibody, further degraded bands ranging from 85-60kDa were observed in control treated cells after Fas activation (Figure 5B) in addition to the 90kDa band observed in Figure 5A, consistent with a serial proteosomal degradation of β -catenin, as previously reported. [10] In CEACAM1-4L expressing cells, these bands showed much lower intensity and the most degraded ~60kDa band was completely absent, after immunoprecipitation in addition to the two major 92/90kDa bands observed in Figure 5A, further supporting increased protection from cleavage after Fas activation. As a control, camptothecin induced apoptosis exhibited no change in the size of β -catenin, suggesting this is a Fas-specific phenotype (Figure 5A and 5B, CPT).

Post-translational modifications of β -catenin are critical to regulate its activity. For example, tyrosine phosphorylation of β -catenin releases the protein to the cytosol where it is subject to proteosomal degradation [10, 22]. When Jurkat cells with and without CEACAM1-4L expression were treated with anti-Fas antibody, control cells but not CEACAM1-4L expressing cells showed tyrosine phosphorylation on β -catenin after 2 hours of anti-Fas stimulation, demonstrating that CEACAM1-4L protected β -catenin from tyrosine phosphorylation (Figure 5B). The degradation and phosphorylation of β -catenin occurred after 2 hrs of anti-Fas treatment but was not observed at 30 min, nor in all time points of camptothecin treatment, indicating phosphorylation, translocation and degradation of β -catenin is a relatively slow process requiring reorganization of the cytoskeleton [10] and is specific for the Fas mediated apoptosis pathway. The role of the cytoskeleton during Fas-mediated apoptosis was investigated later.

Lack of CEACAM1 ITIM involvement in Fas-mediated apoptosis

Since phosphorylation of the ITIMs on CEACAM1-4L followed by recruitment of SHP-1 are the usual mechanism of inhibition of tyrosine phosphorylation on a variety of CEACAM1-4L associated receptors [2], it was important to also determine the phosphorylation status of CEACAM1-4L. It was previously shown that mutation of S508A (S503 in mice) in the cytoplasmic domain of CEACAM1-4L prevents ITIM phosphorylation [23], we also investigated the effect of this mutation on Fas mediated apoptosis in Jurkat cells. As shown In Figure 6A this mutation did not affect the percent apoptosis in Jurkat cells after Fas treatment compared to wild type of CEACAM1-4L. However the S508D mutation, which mimics phosphorylation on S508, increased apoptosis up to 20% higher than wild type cells treated with anti-Fas antibody (Figure 6A). Since the S503A mutation in murine CEACAM1 was postulated to prevent phosphorylation of the ITIMs [23-24], one would expect that the S508D mutation would either enable or increase ITIM phosphorylation. In this respect, our results are somewhat unexpected, suggesting that S508D affects -catenin interactions rather than (just) ITIM activation. In any case, we conclude that the ITIMs on CEACAM1-4L play no obvious role in the protection from apoptosis in this system. In fact, since the opposite result was obtained, it appears that S508 may play a role in the negative regulation of the Arm motif in CEACAM1-4L.

Since the K470A Arm-binding mutant but not wild type CEACAM1-4L inhibited camptothecin mediated apoptosis (Figure 3A), it was also important to test the role of the S508A and S508D mutants in the intrinsic apoptotic pathway. Interestingly, the S508A mutant inhibited as well as the K470A mutant and the S508D mutant restored apoptosis to control or wild type levels (Figure S6). These results indicate that the Arm-binding motif in CEACAM1-4L plays different roles in the extrinsic versus the intrinsic apoptotic pathways and that S508 phosphorylation is a major regulator of the activity of this motif.

When CEACAM1 was immunoprecipitated from CEACAM1-4L transfected cells before or after anti-Fas antibody treatment and western blotted with anti-phosphotyrosine antibody, the levels of phosphotyrosine were low and unchanged compared to a pervanadate treated positive control for tyrosine phosphorylation (Figure 6B). Furthermore, when the CEACAM1 IPs were western blotted for SHP-1, no association between CEACAM1 and SHP-1 was detected (data not shown), indicating that significant activation of CEACAM1's ITIM did not occur during Fas stimulation. In addition, pervanadate treatment accelerated anti-Fas-induced apoptosis in wild type Jurkat cells, reaching 60% apoptosis within 3 hours compared to 4 hours in the absence of pervanadate, as well as reducing the difference between CEACAM1-4L transfected and wild type cells to 20% compared to 50% inhibition in the absence of pervanadate (Figure 6C and D). These results support the finding that tyrosine phosphorylation of β -catenin correlates with and even accelerates its degradation [22] and that the Arm-binding motif of CEACAM1-4L's cytoplasmic domain which inhibits β -catenin degradation and phosphorylation is independent of its ITIMs. Unlike CEACAM1 (Figure 6B), pervanadate treatment did not increase tyrosine phosphorylation of β -catenin, suggesting that the tyrosine phosphorylation of - requires Fas ligation (Figure 5B).

Since previous studies suggest that anti-CEACAM1 antibody treatment of CEACAM1-4L positive T-cells affects their activity via phosphorylation of its ITIM [4], we also tested the effect of anti-CEACAM1 antibody 5F4 in Fas-mediated apoptosis in CEACAM1-4L transfected Jurkat cells or activated PBMCs. In both types of cells, anti-CEACAM1 antibody treatment neither protected nor accelerated apoptosis (Figure S4). These results provide further evidence that the ability of CEACAM1-4L to protect T-cells from either extrinsic or intrinsic apoptotic stimuli does not require activation of its ITIMs.

CEACAM1-4L and β -catenin co-reside in the membrane after Fas activation

As shown previously, CEACAM1-4L and β -catenin co-localize in membranous specks in CEACAM1-4L transfected Jurkat cells and in CEACAM1-4L positive activated T-cells, and a GST-CEACAM1 cytoplasmic domain fusion protein pulls down β -catenin from Jurkat cells [5]. Subcellular fractionation showed that in Jurkat cells the majority of CEACAM1 and β -catenin co-reside in the membrane fraction (Figure 7).

The protection of β -catenin degradation by CEACAM1 is also evident in Figure 7, where, after stimulation, the majority of intact 92kDa β -catenin was in the membrane fraction in CEACAM1 expressing cells, compared to in control treated cells the membrane fraction showed both 92 kDa and the 90kDa degraded bands in lower intensity, the same pattern observed in Figure 5A with un-fractionated cell lysates. The cytosolic fraction of control treated cells showed a significant amount of the 85-60 kDa bands, and both 92 kDa full-length and 90kDa bands were absent, suggesting increased β -catenin degradation in these CEACAM1 deficient cells. In cytosolic fraction of CEACAM1 expressing cells the 90kDa band was observed along with low intensity for the degraded 85-60kDa bands. These same lower molecular weight bands and pattern of protection were also observed in Figure 5B in unfractionated cell lysates when β -catenin signal was enriched by immunoprecipitation. These results suggest that the protective interaction between CEACAM1 and β -catenin occurred in the membrane, in agreement with the previous finding that β -catenin is localized to the cytoskeleton after Fas-stimulation [10].

Therefore, we attempted to co-IP β -catenin with CEACAM1 before and after anti-Fas antibody treatment (Figure S5 and data not shown) to visualize their direct association. In either case, IP of CEACAM1 or IP of β -catenin, the subsequent western blot failed to detect the putative partner before or after 30 min, 2 hrs and 4 hrs of anti-Fas antibody treatment. However, the lack of finding an association in the IP study does not rule out their interaction, since it may be disrupted during the preparation of the cell lysates or the complex might reside in a detergent resistant fraction of the cell that is not accessible by IP analysis.

CEACAM1 expression changes β -catenin's association with actin

Since the β -catenin-CEACAM1 complex could not be captured in a co-IP experiment, it was likely that the interaction was transient and/or involved a further interaction with a common intermediate such as the cytoskeleton. In the case of β -catenin, the β -catenin/actin complex is a crucial component of the adherent junction in epithelial cells [25]. Chung et al [10] have shown that Fas-stimulation promotes β -catenin redistribution and association with the actin cytoskeleton in a detergent resistant compartment. This association plays an essential role in the regulation of β -catenin signaling and apoptosis in hematopoietic cells [10]. In turn, CEACAM1-L also associates with the actin cytoskeleton in a detergent resistant compartment [16].

Therefore, we tested whether CEACAM1 affects the association of β -catenin with actin after Fas stimulation. As shown in Figure 8A, there is little association between β -catenin and actin in Jurkat cells (**upper panel, lane 1**). After stimulation, a obvious increased association occurred between β -catenin and actin, as shown by immunoprecipitation of β -catenin and immunoblotting with actin (**lane 3**), demonstrating the re-distribution of β -catenin to the actin cytoskeleton as previously reported [10]. Immunofluorescent staining of β -catenin in the cytoskeletal detergent resistant fraction of Jurkat cells also exhibited a significant increase after Fas-stimulation (Figure 9 A vs B) as previously reported [10]. However, in Jurkat cells transfected with CEACAM1, co-immunoprecipitation of β -catenin with actin was evident even before stimulation with Fas (Figure 8A, **upper panel, lane 2**),

which agrees with our finding that CEACAM1 inhibits β -catenin signaling in Jurkat cells and promotes its redistribution to the actin cytoskeleton (Figure 9 A vs C). After treatment with anti-Fas antibody for 30 min, the association between β -catenin and actin was diminished in these CEACAM1 expressing cells (Figure 8A, **upper panel, lane 4**) but more evident by cytoskeleton staining (Figure 9 D), suggesting a re-distribution of β -catenin to a detergent resistant pool of actin cytoskeleton. Equal loading and equal immunoprecipitation of β -catenin are shown in Figure 8B and Figure 8A, **bottom panel**, respectively. Since the formation of the β -catenin/actin complex is critical in Fas-mediated apoptosis in Jurkat cells and is responsible for cell shape changes and β -catenin fragmentation [10], regulation of this complex formation by CEACAM1 resulted in inhibition of apoptosis. The fact that CEACAM1 delays but not abrogates apoptosis (Figure 3) also suggests that only a transient interaction with cytoskeleton is involved. Since solubility is a problem for detecting cytoskeleton components by detergents, the failure to detect the β -catenin-actin complex formation after Fas stimulation with a mild detergent NP-40 (data not shown), and to detect the direct association between CEACAM1 and β -catenin by a co-IP (Figure S5 and data not shown) experiment could be explained by proposing that the pool of β -catenin that associates with CEACAM1 resides in a more detergent resistant portion of the cytoskeleton (Figure 7, membrane and pellet fraction and Figure 9 C and D), although their co-localization could be visualized by immunofluorescent staining [5].

Therefore, proof of their interaction is based on four lines of evidence: a) both molecules reside in the same membranous, detergent resistant compartment by either subfractionation, association with actin cytoskeleton (this work) or con-focal analysis [5]; b) the GST-fusion-cytoplasmic domain of CEACAM1-4L specifically pulls-down β -catenin from CEACAM1-4L transfected Jurkat cells [5]; c) transfection of CEACAM1-4L affects both Fas mediated apoptosis and degradation of β -catenin; and d) mutation analysis of the Arm-binding motif in CEACAM1-4L reduces its affinity with β -catenin's Armadillo repeats [5] and abrogates this effect.

In addition, both the long and short isoform of CEACAM1 interact with the actin cytoskeleton [1, 16]. The fact that the short isoform does not contain a β -catenin binding motif but does inhibit Fas mediated apoptosis can now be explained via its modulation of the actin cytoskeleton, which indirectly regulates β -catenin activity. A scheme showing the potential regulation of Fas-mediated apoptosis by β -catenin in the presence and absence of CEACAM1-4L is shown in Figure 10. A summary of the changes in β -catenin regulated by CEACAM1-4L is summarized in Table 1.

Discussion

Our data provides a possible mechanism in which CEACAM1-4L inhibits Fas mediated apoptosis via a novel Arm-binding motif in its cytoplasmic domain. Other than a context-dependent role of potentiating apoptosis or promoting cell survival by β -catenin nuclear signaling [10, 17, 26], our finding supports the idea that CEACAM1 affects T-cell survival by regulation of non-nuclear β -catenin, as shown by prevention of β -catenin degradation and regulation of β -catenin's membrane localization with actin and CEACAM1, as well as a lack of target gene regulation. This also agrees with the previous finding that non-nuclear β -catenin (DN- β -catenin) plays a negative role in Jurkat cell apoptosis [10]. We show that this function is independent of CEACAM1-4L's ITIM activation but depends on its cytoplasmic domain residue S508 whose integrity has been previously shown to be required for ITIM activation [23]. The high expression of β -catenin in leukemic cells, including Jurkat cells, which is associated with resistance to apoptosis [10] can now be re-evaluated in terms of which cells also express CEACAM1, since CEACAM1 is commonly up-regulated in activated T-cells. Besides its role in the inhibition of IL-2 and TCR signaling in the down-

regulation of the T-cell response, the inhibitory role of CEACAM1-4L in T-cells now includes inhibition of Fas-mediated apoptosis. The fact that CEACAM1 transfected Jurkat cells exhibited the same degree of apoptosis as wild type cells after 24 hours of stimulation suggest that the inhibitory effect is a delay in the response, and agrees well with the role of CEACAM1 as a fine-tuning molecule in the immune system [2].

In addition to playing a role in the extrinsic apoptotic pathway, CEACAM1-4L also plays a role in the intrinsic pathway as shown by our camptothecin results. However, in this case, the Arm-motif of CEACAM1-4L plays an opposite role in which only the mutation of this motif K470A or the mutation S508D inhibits camptothecin mediated apoptosis. Combined with the results that suggested there is no obvious ITIM activation in response to Fas stimuli, the various motifs on CEACAM1-4L have distinct and even opposite functions in specific pathways (Figure 9). This may make good biological sense in terms of designating different cytoplasmic motifs for different inhibitory functions.

Finally, it should be noted that the short cytoplasmic domain isoform (CEACAM1-4S) protects Jurkat cells from both apoptotic pathways, but this isoform is mainly expressed in epithelial cells and not in lymphocytes. Thus, the lymphocyte restricted long cytoplasmic domain isoform has inhibitory functions for many of the important signaling pathways studied in these cells. Since the short and long isoform shares the critical residue, F454 for actin-binding [1], this is further evidence that the additional functional motifs on the long isoform such as ITIM or Arm-binding motif may have competing or compensating functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by NIH grant CA 84202.

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1. CEACAM1 inhibits Fas-mediated apoptosis
2. Arm-binding motif in CEACAM1 required for this effect
3. CEACAM1 protects beta-catenin from phosphorylation and degradation
4. ITIM of CEACAM1 not involved in Fas-mediated apoptosis
5. Ser-508 involved in regulation of Fas-mediated apoptosis

Figure 1. Location of key residues in the cytoplasmic domain of human CEACAM1

Cytoplasmic domain of human CEACAM1 short (top) and long (bottom) isoforms are shown with respective key residues. Red: F454, interaction with actin for both short and long isoforms [1, 16]. Blue: H469/K470, interaction with Armadillo-repeat of β -catenin [5]. Orange: Key tyrosines Y493 and Y520 in two ITIMs (underlined) for CEACAM1-L activation [2]. Green: S508, pre-requisite for ITIM activation [23]. Purple: transmembrane domain; Box: non-conserved sequence in the cytoplasmic domain of the short isoform.

Figure 2. CEACAM1 and Fas surface expression in CEACAM1 stable transfected Jurkat cells Jurkat cells (A) and cells transfected with wild type CEACAM-4L (B), and mutants H469A (C) and K470A (D) were stained with anti-CEACAM1-PE and anti-Fas-FITC antibody, respectively. CEACAM1 and Fas expression were determined by FACS and no significant differences of Fas expression were observed. Blue: Isotype Control; Red: CEACAM1-PE/Fas-FITC

Figure 3. Anti-Fas induced apoptosis is inhibited by CEACAM1 expression in Jurkat cells
 Jurkat cells and cells expressing CEACAM1-4L (CEACAM1-4L) and mutations (CEACAM1-4L-H469A, CEACAM1-4L-K470A), as well as CEACAM1-4S (CEACAM1-4S) were treated with 25 or 100 ng/mL anti-Fas antibody at 2, 4 and 24hrs as indicated. Camptothecin (CPT, 10uM) was used to activate the intrinsic apoptotic pathway as a separate control. Apoptosis (**A**) and cell death (**B**) were determined by Annexin V and PI staining, respectively, and analyzed by FACS. Results are representative of >3 experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

Figure 4. Caspase activation is inhibited by CEACAM1 after Fas-stimulation

Jurkat cells were treated with 25 ng/mL anti-Fas for 4hrs and analyzed by western blot or FACS analysis. **(A)** Percent apoptosis of CEACAM1-4L expressing Jurkat cells and control cells were determined by Annexin V staining after 25 ng/mL, 4hrs treatment with anti-Fas antibody, conditions selected for ~50% inhibition in CEACAM1-4L expressing cells. **(B)** Caspase 3 and **(C)** Caspase 8 activities are decreased in CEACAM1-4L expressing cells after Fas-stimulation similar to Annexin V staining, as shown by FACS. Red: Control cells with no treatment. Blue: Cells treated with anti-Fas as in **(A)**. **(D)** Caspase 3/8 cleavage after Fas stimulation as shown by western blotting. The low molecular size bands corresponding to the active forms of Caspase 3/8 are reduced and the high molecular size bands corresponding to pro-Caspase 3/8 are increased in CEACAM1 expressing Jurkat cells after anti-Fas treatment. Numbers denote protection of Caspase 8 activation as pro vs active Caspase 8 levels compared to CEACAM1-4L wild type (100), normalized to Tubulin. Tubulin: loading control.

Figure 5. β -catenin cleavage and phosphorylation after Fas-stimulation

CEACAM1-4L protects β -catenin from degradation (**A**) and phosphorylation (**B**) after Fas-stimulation. (**A**) Cells were treated with anti-Fas antibody and camptothecin for 30min, 2hrs or 4hrs and subject to western blotting for β -catenin and CEACAM1. Internal control: SHP1. (**B**) The same stimulated cell lysates were immunoprecipitated with β -catenin antibody and blotted with β -catenin antibody (upper panel) and anti-phosphotyrosine antibody 4G10 (lower panel). Pervanadate treated cell lysates from CEACAM1 expressing cells were used as a positive control for tyrosine phosphorylation. Note the absence of the full size 92kDa band in (**A**) and presence of the lowest ~50kDa molecular weight bands of β -catenin in (**B**) in control cells after 2hrs of anti-Fas treatment compared to CEACAM1 expressing cells. Also 4G10 blotting for β -catenin is only positive in control cells after 2 hours of treatment (**B**).

Figure 6. Lack of CEACAM1 ITIM involvement in Fas-mediated apoptosis

(A). Cells with the S508A or S508D mutation of CEACAM1-4L were treated with anti-Fas antibody and apoptosis was determined by FACS. The S508D mutation in CEACAM1-4L rescued cell sensitivity to Fas stimulation, whereas the S508A mutation had no significant effect. (B). lack of ITIM phosphorylation of CEACAM1 after 4 hours of anti-Fas stimulation compared to control cell lysate. Stimulated and control cell lysates after 4 hours of anti-Fas treatment were immuno-precipitated with anti-CEACAM1 antibody and immunoblotted with anti-phosphotyrosine antibody, 4G10. The tyrosine phosphatase inhibitor pervanadate treated cell lysate was used as positive control for activation of ITIM and CEACAM1 tyrosine phosphorylation (Perv). No apparent increase in CEACAM1 phosphorylation was observed after Fas stimulation. (C) and (D). Pervanadate accelerates apoptosis and diminishes difference between wild type and CEACAM1 transfected cells. Cells were pretreated with (D) or without (C) pervanadate and stimulated with anti-Fas. Cell apoptosis reached 60% at 3 hours of treatment with pervanadate (D) instead of 4 hours without (C), as determined by Annexin V staining and Caspase 3 activity (data not shown) in cells without CEACAM1 expression. CEACAM1-4L transfected cells are about 45% apoptotic at the time of 3 hrs with pervanadate (D) compared to about 35% at 4hrs without pervanadate treatment (C).

Figure 7. CEACAM1 and β -catenin co-reside in the membrane fraction of Jurkat cells
Control and CEACAM1-4L expressing cells treated with anti-Fas antibody were fractionated by subcellular fractionation kit. β -catenin and CEACAM1 from each fraction was determined by western blotting. Actin was used as loading control. The majority of intact 92kDa β -catenin was in the membrane fraction after treatment in CEACAM1 expressing cells, whereas in control treated cells the membrane fraction showed both 92 and 90kDa bands in less intensity. The cytosolic fraction of control treated cells showed lower molecular weight bands, 85-60 kDa while both the 92 and 90kDa bands were absent. In cytosolic fraction of CEACAM1 expressing cells the 90kDa band was observed with lower amount of the 85-60kDa bands.

Figure 8. CEACAM1 regulates β -catenin and actin association during apoptosis

Control and CEACAM1-4L expressing cells treated with anti-Fas antibody were immunoprecipitated with anti- β -catenin antibody and blotted for actin (**A**, upper panel) or β -catenin (**A**, bottom panel). Association of actin with β -catenin was switched before and after stimulation with anti-Fas between control and CEACAM1 expressing cells. (**B**): Input for actin (upper panel) or β -catenin (bottom panel). Lane 1: control, no treatment; lane 2: CEACAM1, no treatment; lane 3: Control with anti-Fas; lane 4: CEACAM1, anti-Fas.

Figure 9. CEACAM1 expression affects β -catenin cytoskeleton redistribution changed Control and CEACAM1-4L expressing cells treated with anti-Fas antibody were treated with CSK buffer and stained for cytoskeletal β -catenin. Control cells showed significant cytoskeletal β -catenin association after anti-Fas treatment (**B**) vs (**A**), whereas CEACAM1 expressing cells retained cytoskeleton association of β -catenin both before (**C**) and after (**D**) treatment. (**A**) Control cells, no treatment; (**B**) Control cells, with anti-Fas; (**C**) CEACAM1 expressing cells, no treatment; (**D**) CEACAM1 expressing cells, with anti-Fas.

Figure 10. A Model for the regulation of Fas mediated apoptosis by CEACAM1

Left: Anti-Fas activates Fas receptor and triggers the assembly of the FADD complex, which in turn, activates Caspase-8 (Casp 8). β -catenin (β -cat) regulates this process by its recruitment to the cytoskeleton where it is phosphorylated (red) and ultimately degraded by activated Caspases [10]. Right: CEACAM1 expression redirects β -catenin to a distinct actin cytoskeleton pool prior to anti-Fas treatment, slowing the whole apoptotic process.

Table 1Summary of β -catenin state changes during Fas-mediated apoptosis^I

CEACAM1	-	+	-	+
Fas	-	-	+	+
β-catenin phosphorylation	-	-	+	-
β-catenin degradation	-	-	+++	+
Membrane β-catenin degradation	-	-	++	-
Cytosolic β-catenin degradation	-	-	+++	+
Actin association by IP	-	+	+	-
Cytoskeleton β-catenin by IF	-	+	+	+

^IDuring Fas-mediated apoptosis, CEACAM1 regulates β -catenin as follows: 1. Prevention from tyrosine phosphorylation and degradation (Row 1-2); 2. Prevention from degradation in both membrane and cytosolic fractions (Row 3-4); 3. Promotion of association to detergent resistant actin cytoskeleton before stimulation (Row 5-6).