

Death Receptor-3, a New E-Selectin Counter-Receptor that Confers Migration and Survival Advantages to Colon Carcinoma Cells by Triggering p38 and ERK MAPK Activation

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Abstract

E-selectin-mediated adhesion of colon cancer cells to endothelial cells is a key event in metastasis. However, the signaling mechanisms that confer metastatic advantages to cancer cells adhering to E-selectin are ill defined. By using affinity column chromatography and pull-down assays on purified membrane extracts of HT29 and LoVo cells coupled to mass spectrometry analysis, we obtained the first evidence indicating that E-selectin binds to death receptor-3 (DR3) expressed by the cancer cells. Thereafter, we accumulated several results, suggesting that DR3 is an E-selectin receptor on colon cancer cells and that its activation by E-selectin triggers the activation of p38 and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) and confers migration and survival advantages. First, by Western blotting, we found that the E-selectin-binding protein, identified as DR3, is recognized by two anti-DR3 antibodies. Second, the neutralization of DR3 with an antibody and its knockdown by small interfering RNA decrease the adhesion of colon cancer cells to E-selectin and E-selectin-expressing human umbilical vein endothelial cells. Third, inhibiting DR3 and knocking down its expression impair transendothelial migration of HT29 cells and block the activation of p38 and ERK by E-selectin. Fourth, high molecular weight isoforms of DR3 are expressed in samples of primary human colon carcinoma but not in samples from normal colon tissue. Intriguingly, DR3 is a death receptor but its activation by E-selectin does not induce apoptosis in colon cancer cells, except when ERK is inhibited. Our findings identify novel signaling and functional roles of DR3 activated in response to E-selectin and highlight the potential link between DR3 and metastasis. (Cancer Res 2006; 66(18): 9117-24)

Introduction

The adhesion of cancer cells to endothelial cells is a prerequisite for extravasation of circulating cancer cells during metastatic dissemination. It requires specific interactions between adhesion receptors present on vascular endothelial cells and their counter-receptors on cancer cells. E-selectin is a specific endothelial adhesion receptor that is induced by proinflammatory stimuli, such as tumor necrosis factor (TNF)- α and interleukin-1 β (IL-1 β ; 1). The usual role of E-selectin is to mediate the adhesion

of leukocytes to the endothelium allowing their extravasation into inflamed tissues (1). Several lines of evidence indicate that cancer cells parasitize the inflammatory system and interact with E-selectin to extravasate and form metastasis (2–4). In particular, colon carcinoma cells, including HT29 cells, adhere to and roll on both purified E-selectin and cytokine-stimulated endothelial cells *in vitro* (5–7). Moreover, the binding efficiency of clonal colon cancer cell lines to E-selectin is directly proportional to their respective metastatic potential (8). Interestingly, several studies have shown that the adhesion of cancer cells to E-selectin initiates a reverse signaling in the cancer cells, which raises the possibility that this signaling modulates the metastatic potential of cancer cells (9–11).

The binding of cancer cells to E-selectin on endothelial cells requires a molecular scaffold composed of oligosaccharides that are borne by a specific protein backbone on cancer cells (12, 13). This scaffolding complex is known as E-selectin ligand or counter-receptor and is essential for cell binding (4, 14, 15). Furthermore, the expression of these glycosylated proteins correlates with tumor progression and a poor prognosis (16, 17). Sialyl Lewis-x and sialyl Lewis-x are the representative oligosaccharides involved in E-selectin binding (15, 18, 19). In contrast, all studies have failed to identify a functional signaling E-selectin counter-receptor protein. LAMP-1, LAMP-2, and CD44 were identified as E-selectin ligands present on colon cancer cells (20, 21). However, nothing is known about the signaling that emanates from these receptors in the cancer cells bound to E-selectin.

Death receptor-3 (DR3) is a member of the second group of the TNF receptor (TNFR) superfamily, such as TNFR1, DR4, DR5, DR6, and Fas. These receptors are characterized by the presence of a common 70- to 80-amino acid homologous region in the cytoplasmic tail called the death domain (22–27). The signaling pathways induced by these receptors are similar and rely on oligomerization of the receptor by ligand binding, recruitment of death domain proteins, such as TRADD, FAD, or RIP1, through homophilic interaction of their death domains, and subsequent activation of the caspases apoptotic cascade or the transcription factor nuclear factor- κ B (NF- κ B) (28). The sequence homology between DR3 and TNFR1 is especially high, being 40% in the death domain and 22% in the extracellular cysteine-rich domain (25). However, in contrast to TNFR1, ubiquitously expressed, DR3 is preferentially expressed in peripheral blood leukocytes and in lymphocyte-rich tissues, including thymus and spleen, and to a lesser extent in small intestine, colon, fetal lung, and fetal kidney (24, 27). The precise role of DR3 in a physiopathologic context is unclear. However, its ectopic expression in mammalian cells induces apoptosis and activates the transcription factor NF- κ B, depending on the cytoplasmic effectors engaged in the signaling complexes downstream of the death domain (24, 25). Of note, the expression of DR3 is completely suppressed in some transformed

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lymphocyte cell lines as a possible way to avoid apoptosis (26). Analogously, the activation of DR3 by TL1A, an endothelium-derived T-cell costimulator that is the only known ligand for DR3, is not followed by apoptosis in human erythroleukemic TF-1 cells, presumably because it is associated with the expression of the apoptosis-inhibiting protein c-IAP2 (29, 30). In this context, TL1A induces apoptosis in the presence of cycloheximide that blocks the synthesis of c-IAP2.

We report here that DR3 is a new high-affinity functional and signaling sialylated counter-receptor for E-selectin that it is expressed by metastatic colon carcinoma HT29 cells and LoVo cells. Moreover, the high isoforms of DR3 are present in samples of primary colon carcinoma. DR3 contributes to the attachment of both colon cancer cells on purified E-selectin and on E-selectin-expressing endothelial cells. Moreover, its interaction with E-selectin triggers the activation of the prosurvival extracellular signal-regulated kinase (ERK) and promigratory p38 mitogen-activated protein kinase (MAPK) pathways. However, its activation by E-selectin is nonapoptogenic, except in the presence of ERK inhibition. We propose that activation of DR3 by E-selectin acts as a switch that regulates metastasis by allowing colon carcinoma cells to escape apoptosis at the benefit of migratory (p38) and survival (ERK) events.

Materials and Methods

Reagents and antibodies. Recombinant human E-selectin (rhE-selectin)/Fc and TL1A were obtained from R&D Systems (Minneapolis, MN). TNF- α and PD98059 were purchased from Calbiochem (Mississauga, Ontario, Canada). Calcein-AM was obtained from Invitrogen-Molecular Probes (Burlington, Ontario, Canada), and phenylethylisothiocyanate and cycloheximide were from Sigma (St. Louis, MO). Protein A-Sepharose and Agarose wheat germ agglutinin (WGA) column were purchased from Amersham Biosciences (Baie d'Urfé, Quebec, Canada). The following antibodies were used: rabbit anti-DR3 extracellular domain, rabbit anti-DR3 COOH terminus, mouse anti- α -tubulin, and isotype control mouse IgGk (all from Sigma); mouse anti-E-selectin (R&D Systems); rabbit anti-phosphorylated p38/MAPK (T¹⁸⁰/Y¹⁸²), mouse anti-phosphorylated ERK1/2/MAPK (T²⁰²/Y²⁰⁴), and rabbit anti-cleaved caspase-3 (D¹⁷⁵; all from Cell Signaling Technology, Beverly, MA); mouse anti-sialyl Lewis-A (Chemicon, Temecula CA); mouse anti-sialyl Lewis-X (BD Biosciences, Mississauga, Ontario, Canada); and goat anti-mouse IgG (H+L) and goat anti-rabbit IgG (H+L) conjugated with horseradish peroxidase (all from Jackson ImmunoResearch, West Grove, PA). Enhanced chemiluminescence (ECL) was obtained from Pierce Biotechnology, Inc. (Rockford, IL).

Cells. HT29 and LoVo cells were routinely cultivated in McCoy's 5A medium and Ham's medium, respectively, and supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion of umbilical veins from undamaged sections of fresh cords as described (31). The cells were cultured to confluence in gelatin-coated tissue culture flasks in medium 199 containing 20% heat-inactivated FBS, endothelial cell growth supplement (60 μ g/mL), glutamine (2 mmol/L), heparin (25,000 IU), and antibiotics. HUVECs were used at passages \leq 5.

Cell membrane preparation. Cell membranes were isolated from 300×10^6 cells. To preserve intact surface glycoproteins, cells were not harvested with trypsin. After three washes with cold PBS, the cells were scraped and incubated with 1.5 mL sucrose buffer [10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 200 mmol/L sucrose, 2 mmol/L Na₃VO₄, 50 mmol/L NaF, 10 μ mol/L leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)] for 30 minutes on ice to allow cell swelling. Cells were fragmented by 20 seconds of sonication, and then nuclei and cell debris were removed from the homogenates by centrifugation at $900 \times g$ for 10 minutes at 4°C. The supernatant was centrifuged at $140,000 \times g$ for 75 minutes at 4°C (70.1ti rotor, Beckman Coulter Optima LE-80K ultracentrifuge, Mississauga,

Canada). The membrane pellet was solubilized in buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 1 mmol/L PMSF] for a minimum of 1 hour at 4°C. Triton was avoided to preserve potential oligomerized surface receptors.

Affinity column chromatography and mass spectrometry analysis. Purified membrane extracts have been obtained as described above. They were separated into three equal samples that were passed through three separate Agarose WGA columns. The glycosylated proteins grasped by WGA were eluted in a buffer containing 100 mmol/L *N*-acetylglucosamine. The eluates from the three WGA columns were dialyzed for 12 hours using a 14-kDa cutoff dialysis membrane and passed for 12 hours through three separate second columns made of rhE-selectin/Fc coupled to protein A-Sepharose. The proteins that bind to E-selectin were eluted in a buffer containing 10 mmol/L EDTA. The eluates from the three columns were pooled and concentrated to 50 mL by passing through a Microcon membrane YM-30 (Millipore, Bedford, MA). All these steps were done at 4°C. After concentration, the proteins were separated by SDS-PAGE and revealed with SYPRO Ruby. The major band (\sim 200 kDa) was excised, digested with trypsin, and analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MS; SAM Center, Calgary, Alberta, Canada).

Pull-down affinity-binding assay. Pull-down assays were done at 4°C on purified membrane extracts obtained from HT29 and/or LoVo cells. Total proteins (150 μ g) were precleared with 20 μ L protein A-Sepharose (50%, v/v) for 1 hour at 4°C. After centrifugation, supernatant was incubated overnight with 10 μ g rhE-selectin. Molecular complexes were recovered by the addition of 20 μ L protein A-Sepharose for 2 hours at 4°C. After five washes with lysis buffer, the molecular complexes were released from protein A-Sepharose by boiling for 5 minutes with 15 μ L of 4 \times concentrated sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and analyzed in Western blotting using the anti-DR3 COOH-terminal antibody.

Immunoprecipitation. Immunoprecipitation of DR3 has been done on purified membranes extracts obtained from HT29 and LoVo cells as described above. Membrane extracts (150 μ g proteins) were precleared with 20 μ L protein A-Sepharose (50%, v/v) coupled to isotype control mouse IgGk for 1 hour at 4°C. After centrifugation, the supernatant was incubated overnight with anti-DR3 antibody. Molecular complexes were recovered by the addition of 20 μ L protein A-Sepharose for 2 hours at 4°C. After five washes with lysis buffer, the molecular complexes were released from protein A-Sepharose by boiling for 5 minutes with 15 μ L of 4 \times concentrated sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and analyzed in Western blotting.

MAPK activation. p38 and ERK activation has been assayed by Western blotting using antibodies against phosphorylated p38 and phosphorylated ERK. The assays were done on cells that have been incubated in serum-free medium for 16 hours before treatments.

Western blotting. Cells lysis was done in 1% Triton X-100 lysis buffer at 4°C. Then, lysates were centrifuged at $13,000 \times g$ for 15 minutes. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Each antibody was used according to the manufacturer protocol. Blots were then developed with ECL. If necessary, the membrane was reprobated with mouse anti- α -tubulin antibody for normalization.

Adhesion assay. Adhesion assays were done on HUVEC or rhE-selectin/Fc as described previously (32) and indicated in the figure legends. Before assays, cancer cells were harvested and incubated at 37°C for 2.5 hours to regenerate surface glycoproteins (33). Cells were labeled with calcein-AM and left to adhere (10^5 per well) on the endothelial monolayer or on the rhE-selectin matrix for 30 minutes at 37°C. Nonadherent cancer cells were removed and the wells were washed four times with the medium. Attached cells were quantified by measuring the fluorescence emission using a fluorometer.

Cell migration assay. Cell migration was assayed using a modified Boyden chamber assay as described recently (34) and indicated in the figure legends. Before assays, HT29 cells were labeled with calcein-AM and then were added (150×10^3 per Transwell) on the endothelial monolayer or on the rhE-selectin/Fc matrix. The chambers were incubated for 14 hours at 37°C in a 5% CO₂ atmosphere. At the end of incubation, fluorescent

tumor cells that have migrated to the lower face of the filter were counted in five fields under a fluorescent microscope using $\times 20$ magnification.

Cell death and apoptosis assays. Cell death was evaluated by measuring the release of lactate dehydrogenase (LDH) using a cytotoxicity detection kit (Roche, Laval, Canada). Cells were seeded in 96-well microtiter plates to reach 70% confluence after overnight culture. Thereafter, they were treated as indicated in each figure legend and incubated for 24 hours at 37°C. Culture medium from each well was collected and transferred into 96-well plates. LDH activity was determined by following the manufacturer protocol. For apoptosis assays, cancer cells (150×10^3) were plated in 35-mm culture dishes and preincubated in serum-free medium for 16 hours before treatments that were also carried out in serum-free medium. In some conditions, cells were pretreated with 20 $\mu\text{mol/L}$ MAPK/ERK kinase (MEK) inhibitor (PD98059) for 1 hour at 37°C. For caspase analysis, cells were incubated for 6 hours with rhE-selectin/Fc, TL1A, or TNF- α . After 6 hours of treatments, cells were washed and lysed for Western blotting analysis as described above. Cleaved-caspase-3 was analyzed as signaling marker of apoptosis.

Knockdown of DR3 by small interfering RNA. Human DR3 small interfering RNA (siRNA; sense, 5'-CCGUCCAGUUGGUGGUA-3', and antisense, 5'-UUACCACCAACUGGACGG-3') and control siRNA (unrelated gene) were purchased from Qiagen (Mississauga, Ontario, Canada). To silence DR3 expression, HT29 cells were harvested with trypsin, washed, and suspended in serum-free McCoy's medium. A total of 10^6 cells in 400 μL were incubated with 400 pmol DR3 siRNA or control siRNA for 10 minutes at room temperature. Then, HT29 cells were electroporated for 220 μs with 500 V, which was repeated once after 1 minute interval. The electroporated cells were transferred in McCoy's medium with 10% FBS and incubated at 37°C for 48 hours before experiments.

Results

Identification of DR3 as an E-selectin counter-receptor on colon cancer cells. As an initial approach to identify potential signaling receptors for E-selectin on colon cancer cells, we used affinity chromatography done on proteins from purified membrane extracts of HT29 cells. Knowing that E-selectin binds only to glycosylated proteins, extracts were first passed through a WGA column. Then, the glycosylated proteins were passed through a second column containing rhE-selectin/Fc. Using this procedure, we found a major band that migrated at ~ 200 kDa and a faint band that migrated at 66 kDa (Fig. 1A, left). The 200-kDa band was identified as DR3 by MS. The analysis revealed three peptides related to DR3 that cover 38% of its death domain sequence (Fig. 1A, right). We next confirmed the identification of DR3 as an E-selectin counter-receptor by pull-down assays done on membrane extracts of HT29 and LoVo cells and using rhE-selectin/Fc as ligand. We found three major bands that migrated at ~ 180 , 66, and just >45 kDa (Fig. 1B). Both together, the findings of our column chromatography and pull-down approaches are strong indications that DR3 is a bona fide counter-receptor for E-selectin.

DR3 exists at least under 11 distinct isoforms generated by alternative splicing. The major isoform has a molecular weight of 47 kDa (25). Accordingly, in total extracts from HT29 and LoVo cells, we found in Western blot several bands that are recognized by anti-DR3 antibodies directed either against the extracellular domain (Fig. 1C) or the intracellular domain of DR3 (data not shown). This includes the major isoform at 47 kDa and other bands migrating at approximately 180, 97, 66, and 55 kDa (Fig. 1C). Given that E-selectin binds only to proteins that bear sialyl Lewis oligosaccharides, we found that both sugars were present on DR3 from LoVo and HT29 cells (Fig. 1D). In particular, the sialyl Lewis-a was found on the 200-kDa DR3 band from both types of cells (Fig. 1D). Of great significance, we found that a high isoform of

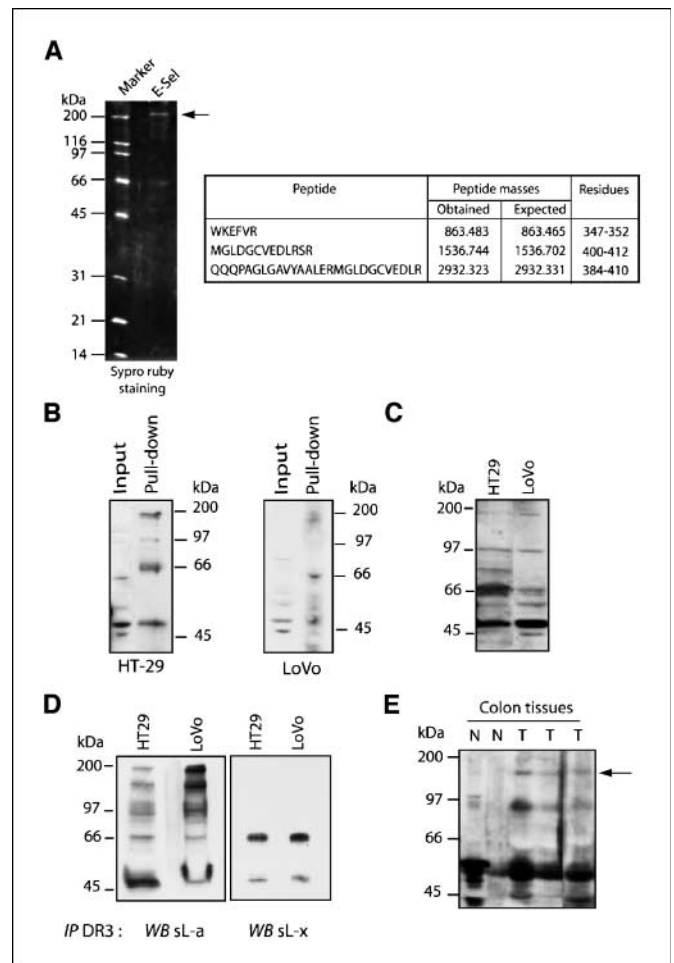


Figure 1. Identification of DR3 as an E-selectin counter-receptor on colon cancer cells. *A, left*, E-selectin (*E-Selectin*)-binding glycoproteins were isolated from membrane extracts of HT29 cells by affinity chromatography using a WGA column as a first step followed by rhE-selectin/Fc column as a second step. MS analysis of the ~ 200 -kDa band (*arrow*) revealed three peptides related to DR3 and that covers $\sim 38\%$ of its death domain sequence. *Right*, the tryptic peptide ions obtained were scanned against the Swiss-Prot and Genpep databases using the MS-FIT program of Protein Prospector. *B*, pull-down affinity-binding assay with rhE-selectin/Fc was done on membrane extracts (150 μg) from HT29 and LoVo cells. The presence of DR3 bound to E-selectin was revealed by an anti-DR3 antibody directed against the COOH-terminal domain of DR3. Three major bands migrating at ~ 180 , 66, and ~ 47 kDa were detected. Inputs correspond to 5% of the membrane extract. *C*, Western blot analysis of DR3 in total extract (25 μg) of HT29 and LoVo cells confirmed the presence of the major isoform at ~ 47 kDa and other bands migrating at ~ 180 , 97, 66, and ~ 55 kDa. *D*, immunoprecipitation of DR3 (*IP DR3*) from total extracts followed by Western blot against sialyl Lewis-a (*WB sL-a*) and sialyl Lewis-x (*WB sL-x*) showed that DR3 bears sialyl Lewis-a and sialyl Lewis-x oligosaccharides in a similar pattern in both colon cancer lines HT29 and LoVo. *E*, Western blot analysis of DR3 in samples of normal (*N*) and primary tumor (*T*) of colon tissues revealed the presence of a major isoform at ~ 55 kDa in all the samples and an ~ 150 -kDa higher isoform that was present only in primary tumor samples (*arrow*).

DR3 migrating at about 150 kDa is expressed in primary colon carcinoma samples but not in normal colon tissues (Fig. 1E). Taken together, these data show for the first time that DR3 expressed by colon carcinoma cells is a high-affinity sialylated receptor for E-selectin.

DR3 mediates the attachment of HT29 and LoVo cells to purified E-selectin and E-selectin-expressing HUVEC. To determine whether the expression of DR3 by HT29 and LoVo cells regulates their attachment to E-selectin, we first did adhesion

assays of these cells on rhE-selectin/Fc or on HUVEC that express E-selectin in the presence or absence of blocking antibodies. Results showed that the anti-DR3 antibody reduced by 30% and 50% the adhesion of HT29 and LoVo cells, respectively, to rhE-selectin/Fc. In contrast, an anti-E-selectin antibody reduced by 90% and 70% the adhesion of HT29 and LoVo cells, respectively, to E-selectin (Fig. 2A). Similar results were obtained when the colon cancer cells were added to E-selectin-expressing HUVEC (Fig. 2B). In fact, we found that cancer cells adhered poorly to control monolayers of HUVEC, whereas the adhesion capacity was increased 4-fold on HUVEC that expressed E-selectin. In the presence of anti-E-selectin-neutralizing antibodies, only ~45% of HT29 and 55% of LoVo cells adhered to HUVEC, confirming that E-selectin is a major receptor for colon cancer cells on the endothelium. Moreover, pretreating HT29 and LoVo cells with the anti-DR3 antibody reduced by 30% and 45%, respectively, their adhesion to E-selectin-expressing HUVEC, supporting the involvement of DR3 as an E-selectin counter-receptor (Fig. 2B). To further highlight the role of DR3, we knockdown its expression by transfecting siRNA that targets human DR3 mRNA. As shown in Fig. 2C, the silencing approach resulted in a 50% decrease in the expression of the major DR3 isoform. The knockdown of DR3 was associated with a 30% reduction of the adhesion of HT29 cells to rhE-selectin/Fc or to E-selectin-expressing HUVEC. In contrast, an irrelevant siRNA does not affect cell adhesion (Fig. 2D). Because E-selectin binds only to glycosylated ligands bearing sialyl Lewis-a and sialyl Lewis-x, we found that antibodies directed against these oligosaccharides showed a similar level of inhibition as that of the anti-DR3 antibody on cancer cell adhesion to E-selectin (Fig. 2A and B). This is consistent with the fact that the anti-DR3 antibody impairs the interaction between DR3 and E-selectin because it targets the extracellular amino acids 59 to 77 of which N67 is one of the two sites of N-glycosylation.

Together, these findings indicate that DR3 is a sialylated glycoprotein that participates to the adhesion of colon cancer cells to E-selectin. However, DR3 is not probably the sole E-selectin counter-receptor that is involved in the adhesion process because its neutralization and knockdown did not abolish the binding of cancer cells to E-selectin.

E-selectin binding to DR3 mediates activation of p38 and ERK in colon cancer cells. The interaction between HT29 cells and E-selectin activates the p38 and ERK pathways in the cancer cells (11, 32). We thus examined whether the E-selectin-mediated activation of these pathways results from its interaction with DR3. HT29 cells were treated for 5 and 30 minutes with rhE-selectin/Fc, and then p38 and ERK activation was measured. Results showed that rhE-selectin/Fc increased by ~2-fold the activation of p38 and by 1.5-fold that of ERK1/2 after 5 minutes (Fig. 3A). For ERK, the activation reached 4.5-fold after 30 minutes but remained close to basal level for p38. As expected, pretreatment of cancer cells with a heat-inactivated form of rhE-selectin/Fc was inefficient in activating the kinases, indicating that it was a relevant negative control. Most importantly, the activation of both MAPKs was markedly reduced when colon cancer cells were pretreated with an anti-DR3 antibody or in the presence of an anti-E-selectin-neutralizing antibody. In contrast, pretreatment of cancer cells with an irrelevant antibody did not inhibit the activation of the MAPKs by E-selectin, and the anti-DR3 antibody did not activate by itself p38 or ERK (Fig. 3A, right and Fig. 3B, right). Along the same lines, the knockdown of DR3 with siRNA resulted in an impairment in the activation of p38 and ERK in HT29 cells (Fig. 3A,

right and Fig. 3B, right). Interestingly, the combined addition of antibodies directed against both sialyl Lewis-a and sialyl Lewis-x led to a 80% to 100% inhibition of p38 and ERK activation induced by E-selectin. These results show that DR3 is a major E-selectin-sialylated signaling counter-receptor upstream of p38 and ERK activation in HT29 cells.

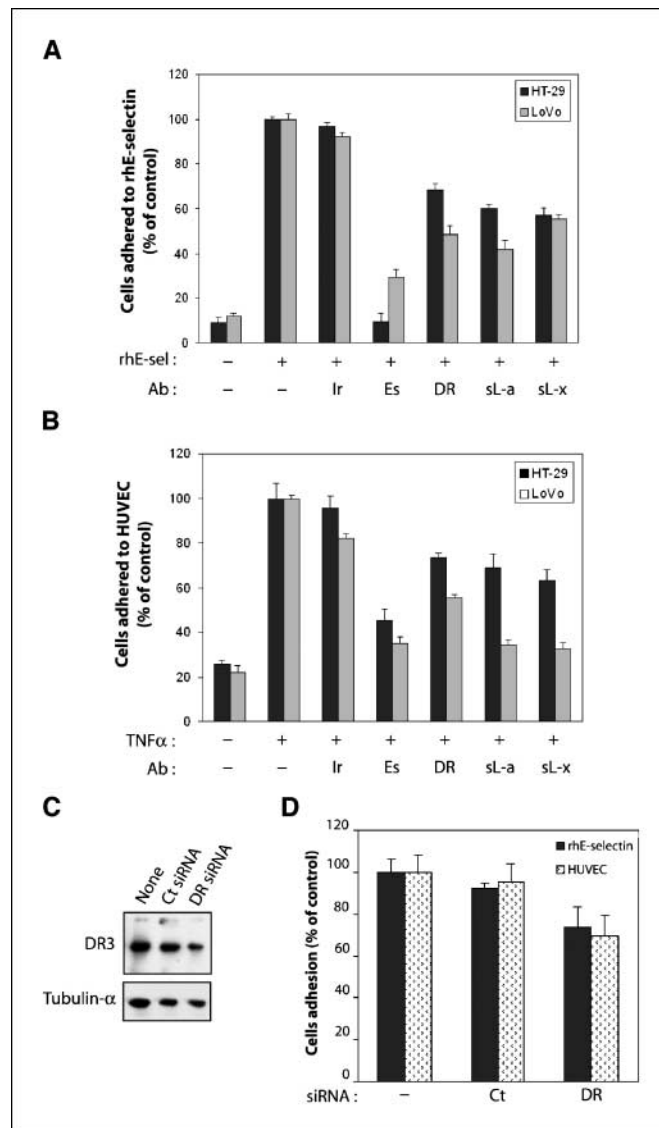


Figure 2. DR3 mediates the attachment of HT29 and LoVo cells to purified E-selectin and E-selectin-expressing HUVEC. Adhesion assays were done on rhE-selectin/Fc (rhE-sel; A and D) or on E-selectin-expressing HUVEC (B and D) as follows. For the assays done on HUVEC, the cells were plated on gelatin-coated 96-well microtiter plates and grown to confluence for 24 hours. The expression of E-selectin at the surface of HUVEC was induced by an exposure of 4 hours to TNF- α (10 ng/mL). Then, the medium was removed and replaced by fresh medium. For the assay on rhE-selectin/Fc, this latter was absorbed to 96-well microtiter plates overnight at 4°C at a concentration of 100 ng/well in TBS. Before adhesion, cells were incubated for 30 minutes with indicated antibodies (Ab) at 10 μ g/mL: irrelevant (Ir), anti-E-selectin (Es), anti-DR3 antibody directed against the extracellular domain (DR), anti-sialyl Lewis-a (sL-a), and anti-sialyl Lewis-x (sL-x). Adhesion assays were carried out after 30 minutes C, DR3 knockdown in HT29 cells transfected with DR3 siRNA (DR siRNA) compared with HT29 cells transfected with negative control siRNA Alexa Fluor 488 (Ct siRNA) as described in Materials and Methods. D, adhesion assays conducted 48 hours after transfection of siRNA show that the knockdown of DR3 is associated with a decreased adhesion of HT29 to both rhE-selectin/Fc and E-selectin-expressing HUVEC. Columns, mean of four independent experiments done in triplicates; bars, SD.

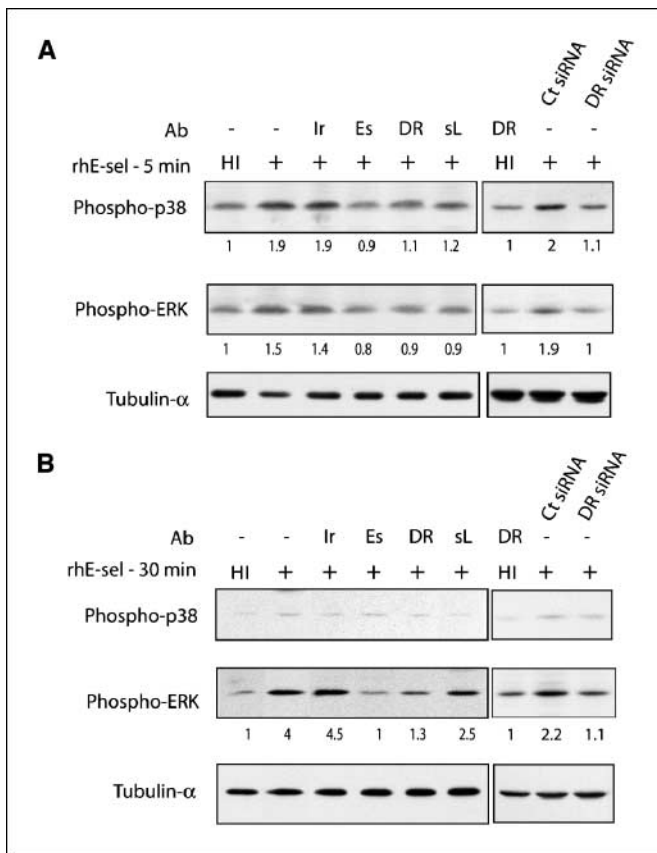


Figure 3. E-selectin binding to DR3 mediates activation of p38 and ERK in colon cancer cells. HT29 cells were treated with rhE-selectin at 5 μ g/mL for 5 (A) or 30 (B) minutes. Before treatments, cells were incubated for 30 minutes with indicated antibodies at 10 μ g/mL: irrelevant, anti-E-selectin, anti-DR3 antibody directed against the extracellular domain, and anti-sialyl Lewis-x (sL). ERK and p38 activation was analyzed by Western blotting using antibodies against phosphorylated p38 and phosphorylated ERK, respectively. Heat-inactivated rhE-selectin (HI) was used as a negative control. A, right and B, right, the effect of DR3 knockdown was analyzed on E-selectin-induced p38 and ERK activation. HT29 cells were transfected with DR3 siRNA or with negative control siRNA as described in Materials and Methods. MAPK activation was analyzed 48 hours after transfection. Equal protein loading was confirmed by reprobing with an anti-tubulin- α antibody. Results show that DR3 is a sialylated signaling receptor for E-selectin that induces p38 and ERK activation. Representative autoradiograms of four independent experiments.

DR3 activation promotes transendothelial migration of HT29 cells adhering to E-selectin. Transendothelial migration of HT29 cells is tightly associated with an increased in their motile potential that results from the activation of p38 in the cancer cells bound to E-selectin (11). Because activation of DR3 is involved in triggering the activation of p38 in HT29 cells, we verified whether DR3 modulates the transendothelial migration of these cells. This was evaluated by determining the capacity of HT29 cells pretreated or not with anti-DR3 blocking antibody or transfected with DR3 siRNA to traverse a layer of E-selectin-expressing HUVEC in a Boyden chamber. We found that the anti-DR3 antibody or the knockdown of DR3 with siRNA inhibited by 66% the increase in transendothelial migration of HT29 cells that is associated with the expression of E-selectin. We also found that pretreating E-selectin-expressing HUVEC with anti-E-selectin antibody almost completely abolished the increase in transendothelial migration of HT29 cells, which supports the essential role played by E-selectin in the process (Fig. 4A). In this context, the migration of HT29 cells in the

Boyden chamber assay was increased by 9-fold when cells were added on E-selectin-coated membranes compared with bovine serum albumin (BSA)-coated membranes. Moreover, the migration of cells, in which DR3 was knocked down with siRNA, was reduced by 30% (Fig. 4B). Overall, these data suggest that activation of DR3 by E-selectin is importantly involved in conferring motility to cancer cells and thereby may confer a metastatic advantage to these cancer cells.

DR3-mediated cell death and apoptosis is not functional in colon carcinoma cells activated by E-selectin. Overexpression of DR3 in several cell lines is associated with induction of apoptosis (22–25). We thus verified whether E-selectin-mediated activation of DR3 triggers cell death in colon cancer cells. LoVo cells were

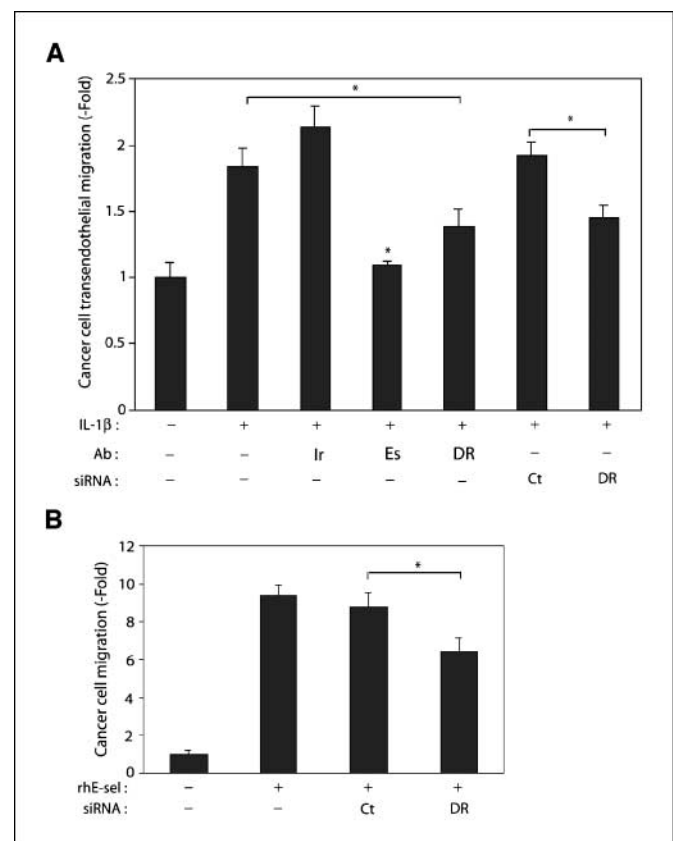


Figure 4. DR3 activation promotes transendothelial migration of HT29 cells adhering to E-selectin. A, transendothelial migration of cancer cells was assayed using a modified Boyden chamber assay. HUVECs were grown to confluence on 8.0- μ m pore size gelatinized polycarbonate opaque membrane and then treated or not with 20 ng/mL IL-1 β for 4 hours at 37°C to induce the expression of E-selectin. Before migration assay, HT29 cells were incubated for 30 minutes with indicated antibodies at 15 μ g/mL: irrelevant, anti-E-selectin, and anti-DR3 antibody directed against the extracellular domain. Transendothelial migration of cancer cells was carried out after 14 hours. The effect of knocking down DR3 was evaluated 48 hours after transfection (DR3 siRNA and negative control siRNA). Results show that the neutralization of DR3 with an anti-DR3 antibody or its knockdown with siRNA inhibits by 66% the increase in transendothelial migration that depends on E-selectin expression by HUVEC. B, effect of rhE-selectin/Fc on cancer cell motility was assayed also using 8.0- μ m pore size membrane coated with 5 μ g/mL rhE-selectin/Fc and then blocked with BSA. Results show that rhE-selectin/Fc increased by 9-fold HT29 cell migration compared with bovine BSA-coated membranes. This effect was reduced by \sim 30% with HT29 cells, in which DR3 was knocked down with siRNA. Columns, mean of two independent experiments done in triplicates; bars, SD. *, $P < 0.05$, statistically significant, difference between control and anti-E-selectin condition or anti-DR3 antibody directed against the extracellular domain condition.

exposed to 5 $\mu\text{g}/\text{mL}$ rhE-selectin/Fc. Thereafter, cell death was evaluated by measuring the release of LDH (35). Results showed that rhE-selectin/Fc decreased by 20% the release of LDH by LoVo cells. This indicates that E-selectin did not induce death in these cells (Fig. 5A). In contrast, the release of LDH was increased by TL1A, TNF- α , and by phenylethylisothiocyanate. As E-selectin binding to DR3 induced a strong activation of ERK, we next examined whether the activation of ERK might be involved in protecting colon cancer cells against the induction of cell death. LoVo cells were pretreated with PD98059 to inhibit ERK activation and then treated with rhE-selectin/Fc, TL1A, or TNF- α . We found that ERK inhibition was associated with an increase in LDH release in response to rhE-selectin/Fc. In the presence of E-selectin, the level of LDH release passed from 20% below basal level in control conditions to 20% over the basal level when ERK was blocked. The addition of cycloheximide to LoVo cells, in which ERK was inhibited, did not affect the release of LDH. Comparable results were obtained with HT29 cells (data not shown).

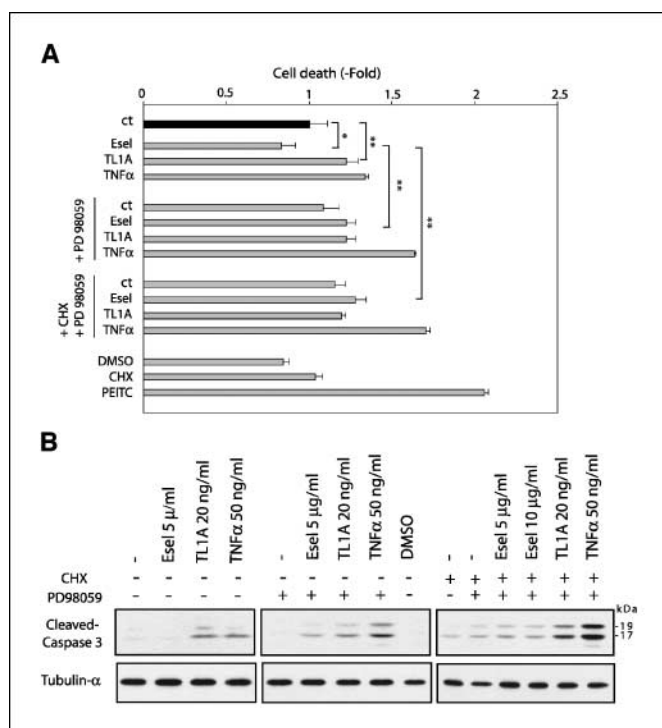


Figure 5. DR3-mediated cell death and apoptotic response is not functional in colon carcinoma cells activated by E-selectin. **A**, LoVo cells were left untreated or pretreated for 1 hour with 20 $\mu\text{mol}/\text{L}$ ERK inhibitor (PD98059) with or without 10 $\mu\text{g}/\text{mL}$ cycloheximide (CHX; both diluted in DMSO) before being incubated with either rhE-selectin/Fc (Esel; 5 $\mu\text{g}/\text{mL}$), TL1A (20 ng/mL), TNF- α (50 ng/mL), or phenylethylisothiocyanate (PEITC; 50 $\mu\text{mol}/\text{L}$). After 24 hours, the release of LDH by damaged cells was evaluated by spectrophotometry at 490 nm as described by the manufacturer's protocol. Results show that activation of DR3 by E-selectin does not lead to cell death in LoVo cells. **Columns**, mean of three independent experiments done in triplicates; **bars**, SD. *, $P < 0.05$; **, $P < 0.01$, statistically significant, difference between indicated conditions. **B**, LoVo cells were left untreated or pretreated for 1 hour with 20 $\mu\text{mol}/\text{L}$ PD98059 with or without 10 $\mu\text{g}/\text{mL}$ cycloheximide before being incubated with either rhE-selectin/Fc (5 or 10 $\mu\text{g}/\text{mL}$), TL1A (20 ng/mL), or TNF- α (50 ng/mL). Western blot analysis of caspase-3 activation (p19 and p17 cleaved caspase-3 fragments) was done 6 hours after treatment. Equal protein loading was confirmed by reprobing with an anti-tubulin- α antibody. Results show that activation of DR3 by E-selectin does not lead to apoptosis in LoVo cells and that ERK activation downstream of DR3 may play a protective function against cell death. Representative autoradiograms of three independent experiments.

Cells death induced by TNF- α and phenylethylisothiocyanate in colon cancer cells is typically apoptotic (36, 37). Accordingly, TNF- α and phenylethylisothiocyanate led to DNA fragmentation in colon cancer cells (data not shown). Moreover, TNF- α induced caspase-3 activation, and the effect was potentiated by inhibiting ERK either in the presence or absence of cycloheximide (Fig. 5B). In contrast, E-selectin did not lead to nuclear fragmentation (data not shown) or activation of caspase-3 (Fig. 5B). However, a weak increase in caspase-3 activation was observed in the presence of PD98059. On the other hand, TL1A activates caspase-3 and the effect was not increased when ERK was blocked, except in the presence of cycloheximide (Fig. 5B). Overall, these results suggest that activation of DR3 by E-selectin does not lead to cell death and apoptosis in colon cancer cells and that ERK activation downstream of DR3 may play a protective function against cell death.

Discussion

Several studies have identified E-selectin as a key endothelial receptor responsible for the adhesion of many types of cancer cells and thereby metastasis (8, 38–41). Moreover, it has been reported that the adhesion of cancer cells to E-selectin initiates a reverse signaling in the cancer cells that may modulate their metastatic potential (9–11). However, no study has identified any signaling event that emanates in cancer cells from the binding of E-selectin to known counter-receptors, such as CD44, LAMP1-2, and ESL-1 (20, 21, 38). The major contribution of our study is to have identified for the first time DR3 as an E-selectin signaling receptor present on two different colon cancer lines that have metastatic characteristics as well as in extracts obtained from primary human colon carcinoma.

The biochemical identification of DR3 as an E-selectin receptor present on the surface of colon cancer cells was first obtained by affinity chromatography done on purified membrane preparations of HT29 cells. It allowed us to isolate a band at 200 kDa that was identified by MS as DR3 with three peptides that cover 38% of the death domain of this receptor. Thereafter, by using pull-down assays done on membrane preparations from HT29 and LoVo cells, we found that E-selectin binds to three major bands at 180, 66, and 47 kDa, and all of these bands correspond to DR3 isoforms because they are recognized by two different anti-DR3 antibodies. Given that E-selectin binds only to proteins that bear sialyl Lewis (4, 15), we found that sialyl Lewis-a was associated with all the DR3 isoforms that bind E-selectin in pull-down assays, whereas sialyl Lewis-x comigrate with the 66-kDa isoforms. This indicates that these DR3 isoforms are appropriately glycosylated for E-selectin binding and that they may be involved in the binding of HT29 or LoVo cells to E-selectin on endothelial cells. Apparently, the high molecular weight isoforms of DR3 correspond to glycosylated oligomers of DR3 because these oligomers are known to resist to SDS denaturation (25). Interestingly, a high molecular weight isoform of DR3 is differentially expressed by samples from primary colon cancer compared with normal colon tissue, which suggests that it is likely involved in the binding of circulating cancer cells to endothelial cells.

The involvement of DR3 as a receptor that participates to the adhesion of colon cancer cells to E-selectin is supported by the findings that the neutralization of DR3 with a blocking antibody and its knockdown with siRNA decrease the adhesion of HT29 and LoVo cells to both E-selectin-expressing HUVEC as well as to

rhE-selectin/Fc. Incidentally, the fact that similar results were obtained either on cancer cells adhering to HUVEC expressing E-selectin and to rhE-selectin/Fc validates the suitability of using rhE-selectin/Fc, a disulfide-linked homodimer, as a model that mimics activated E-selectin on HUVEC. Interestingly, sialyl Lewis-a and/or sialyl Lewis-x, the major oligosaccharides that allow binding of colon cancer cells to E-selectin, are present on DR3. Accordingly, the adhesion of both HT29 and LoVo cells to endothelial cells is markedly impaired by antibodies directed against these sugars. Intriguingly, neutralization of DR3 or its knockdown does not completely inhibit the adhesion of HT29 cells to HUVEC, which suggests the involvement of other E-selectin counter-receptors. In this context, apparently, other E-selectin ligands, such as those identified on other cell lines (LAMP-2, CD-44, and ESL-1), participate with DR3 to the binding of HT29 and LoVo cells to E-selectin.

The finding that the activation of DR3 by E-selectin initiates a reverse signaling in the cancer cells is a major accomplishment. This is supported by the observation that exposure of HT29 cells to rhE-selectin/Fc is associated with an enhancement in ERK and p38 activation and that the increase is completely inhibited by an anti-DR3 neutralizing antibody and by the knockdown of DR3 by siRNA. The inhibition of the E-selectin-induced p38 and ERK activation by these complementary approaches suggests that DR3 is the major E-selectin signaling counter-receptor present on colon cancer cells. Interestingly, the activation of the p38 and ERK kinases by E-selectin is abolished by the combination of both anti sialyl Lewis-a and sialyl Lewis-x, supporting the point that sialylation of DR3 is a prerequisite for binding and signaling. We do not know at the present time the signaling cascades that connect DR3 to p38 and ERK. The possibility that both pathways are activated following classic cascades involving the RIP kinases downstream of the TNF receptors should be considered (42). Intriguingly, DR3 is the sole member of the death receptor family that contains an immunoreceptor tyrosine-based activation motif (ITAM) within its death domain with two conserved tyrosine residues (Y376 and Y394; ref. 43). The binding of ligands to immunoreceptors triggers the phosphorylation of their ITAM motif via the activation of associated tyrosine kinases. In turn, this allows the recruitment of adaptor proteins, which promotes MAPK activation (44, 45). It is then possible that the unique ITAM motif within DR3 is involved in signaling to ERK and p38 in response to E-selectin.

Another important novelty of our study is to have obtained evidence suggesting that signaling from DR3 in the cancer cells is a prerequisite for their transendothelial migration. Notably, we found the binding of HT29 cells to DR3 is required to trigger transendothelial migration of HT29 cells because the process is impaired by blocking DR3 or following its knock down. As discussed above, the adhesion of HT29 cells to E-selectin does not rely only on DR3. In contrast, the signaling that emanates from the binding of HT29 cells to rhE-selectin/Fc relies mainly on DR3. Hence, our results suggest that the marked inhibition of transendothelial migration by the anti-DR3 antibody or DR3 siRNA depends on an inhibition of its signaling in HT29 cells rather than their nonadhesion to HUVEC. Along these lines, we reported previously that transendothelial migration requires an increased motile potential of HT29 cells that needs the activation of the p38 pathway by E-selectin, whereas their adhesion is p38 independent (11). Thus, our findings suggest that DR3-mediated activation of p38 in the cancer cells bound to E-selectin might be a prerequisite

for their transendothelial migration. In this context, binding of E-selectin to DR3 on colon cancer cells might, at least partially, underlies their metastatic potential because metastasis is often associated with an increase motile potential of the cancer cells as well as their ability to cross the endothelium (39, 46). In line with this, we show that DR3-mediated migration of cancer cells is increased when cells are added to E-selectin-coated membrane, which further highlights the role of E-selectin as a signaling endothelial adhesion ligand involved in initiating cancer cell migration. However, the addition of rhE-selectin/Fc in the lower part of the Boyden chamber did not trigger cell migration, indicating that it is not a chemoattractant (data not shown).

DR3 is a death receptor and its activation by TL1A, its cognate ligand, increases LDH release and leads to activation of caspase-3 and to nuclear fragmentation in HT29 cells. In contrast, E-selectin, in concentrations that activate the p38 and ERK pathways, reduces the release of LDH down below basal level, does not activate caspase-3, and does not increase nuclear fragmentation over basal level. This suggests that the receptor has developed signaling pathways that allow escaping cell death and apoptosis in response to E-selectin. Accordingly, the inhibition of ERK partially restores death and apoptosis in colon cancer cells treated with E-selectin, indicating that the activation of ERK has a protective function. Several possibilities may be advanced to explain the protective function of ERK. The fact that the apoptotic response is restored in the presence of PD98058 is an indication that the protective function of ERK occurs downstream of MEK1. In neutrophils, the activation of MEK1-ERK axis by granulocyte macrophage colony-stimulating factor confers protective function against Fas-induced apoptosis by impairing the recruitment of FADD to Fas (47). Given that the apoptotic functions of DR3 are tightly associated with the recruitment of FADD (22, 25), a likely possibility is that E-selectin-mediated activation of ERK protects against apoptosis by impairing the recruitment of FADD to DR3. On the other hand, ERK activation leads to phosphorylation of DR3 on cytoplasmic serine residues, which might confer protection against apoptosis (48). Hence, apparently, E-selectin-mediated activation of ERK triggers a loop that inhibits apoptosis by an unknown mechanism but that depend on the phosphorylation of serine residues within DR3. Incidentally, it is interesting to mention that the apoptotic effect of TNF- α is also increased by PD98059 and that its receptor is the only other member of the family that is phosphorylated on serine through the ERK pathway. Moreover, our finding that cycloheximide increases the apoptotic response associated with TNF- α or TL1A in cells, in which ERK is blocked, may be explained by the fact that cycloheximide inhibits newly synthesized proteins playing a role in apoptosis blockage (30, 49). Intriguingly, despite its ability to activate ERK on short periods (30), TL1A-induced apoptosis is independent of ERK because it is not affected by PD98059, except in the presence of cycloheximide. A possible explanation is that the pattern of glycosylation required for E-selectin signaling might not be essential for TL1A. In that regard, an increase in the level of sialylation of the death receptor CD95 (APO-1/Fas) has been proposed as an antiapoptotic mechanism that converts tumor cells to a more malignant phenotype (50).

Overall, our study is the first to identify DR3 as a signaling receptor for E-selectin on colon cancer cells. Moreover, the fact that activation of DR3 in response to E-selectin triggers transendothelial migration of cancer cells and protects them against apoptosis raises the possibility that DR3 has evolved to provide

metastatic advantages to colon cancer cells. Interestingly, E-selectin released by endothelial cells is associated with poor overall survival for colon cancer, which possibly results from its binding to DR3 on circulating cancer cells (51). Altogether, our new findings open important new avenues in understanding colon cancer metastasis and may lead to the development of new strategies to fight this dreadful complication of cancer.

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Death Receptor-3, a New E-Selectin Counter-Receptor that Confers Migration and Survival Advantages to Colon Carcinoma Cells by Triggering p38 and ERK MAPK Activation

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