

TRAIL inhibits platelet-induced colorectal cancer cell invasion

Journal of International Medical Research

2019, Vol. 47(2) 962–972

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DOI: 10.1177/0300060518820785

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Abstract

Objective: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a pro-apoptotic ligand that activates the extrinsic apoptosis pathway of cell death receptors. This study aimed to evaluate the relationship between TRAIL and platelet-induced tumor metastasis in colorectal cancer.

Methods: Platelet P-selectin (CD62P) was measured by immunohistochemistry in tumor and adjacent normal tissues from 90 patients with colorectal cancer undergoing resection. Tumor cell invasion was assessed by transwell assay in the presence of platelets with or without TRAIL. The expression of TRAIL receptors DR4 and DR5 on platelets was assessed by flow cytometry, real-time polymerase chain reaction, and western blotting.

Results: P-selectin (CD62P) expression was significantly increased in tumor tissues compared with adjacent normal tissues. High CD62P expression was significantly correlated with tumor stage and vascular invasion. Tumor cell migration was increased by coculture with platelets, but this effect was inhibited by TRAIL. Transforming growth factor (TGF)- β 1 secretion was significantly reduced in TRAIL-treated platelets. The TRAIL receptor DR5 but not DR4 was expressed in platelets according to flow cytometry.

Conclusions: TRAIL could inhibit metastasis and colon cancer cell invasion by promoting platelet apoptosis and reducing the release of TGF- β 1.

Keywords

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), platelet, colorectal cancer, CD62P, metastasis, transforming growth factor- β 1

Date received: 14 October 2018; accepted: 2 December 2018

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Introduction

Platelets are known to play key roles in tumor growth and metastasis, and patients with solid tumors, most commonly primary colorectal, lung, breast, or gastrointestinal tumors, often have thrombocytosis.¹ P-selectin (CD62P) is a cell adhesion molecule that is abundantly expressed in activated platelets.² A clinical study showed that the number of platelets was significantly higher in patients with malignant tumors compared with normal individuals and patients with benign tumors, suggesting that platelets may participate in tumor occurrence and development.³ Furthermore, Liang et al.'s study indicated that activated platelets might stimulate metastasis of colon cancer cells, and inhibition of platelet activity might thus be a novel method of minimizing the risk of metastasis during surgery.⁴ However, strategies for inhibiting platelet activation are lacking. Further studies showed that platelets could interact with tumor cells to form tumor emboli, which could evade immune surveillance and promote tumor metastasis.⁵ The mechanism responsible for this process may involve three steps: 1) tumor cells activate platelets and form a tumor thrombus, thereby protecting tumor cells from immune system attacks; 2) the platelet surface uses adhesion molecules to bind to tumor cells and vascular endothelial cells, thus helping tumor cells implant at the metastatic site; and 3) platelets can promote tumor growth and angiogenesis by secreting a variety of bioactive factors to provide a suitable microenvironment for tumor growth. Specifically, transforming growth factor (TGF)- β secreted by platelets can promote tumor metastasis^{6,7} by regulating cell growth.^{8,9}

Tumor necrosis factor related apoptosis-inducing ligand, also known as Apo lipoprotein 2 ligand (TRAIL/Apo2L), is a member of the tumor necrosis factor superfamily and a type II transmembrane protein.¹⁰

TRAIL is expressed in different cells of the immune system and plays a role in tumor monitoring and suppression by T cells and natural killer cells.¹¹ Five types of TRAIL receptors have been identified to date: death receptors DR4 and DR5, 'decoy' receptors DcR1 and DcR2, and a fifth soluble receptor, osteoprotegerin.¹² Previous studies found that recombinant TRAIL protein inhibited the growth of tumor cells and even caused tumor regression, with no obvious damage to the host, suggesting potential prospects for its application in the treatment of tumors. One study showed that TRAIL could inhibit tumor metastasis by inducing tumor cell apoptosis.¹³ Other researchers found that TRAIL-induced miR-146a expression suppressed human breast cancer migration mediated by the chemokine receptor CXCR4, which was highly correlated with cancer metastasis and was a key gene controlling chemotaxis of tumor cells.¹⁴ These findings suggest that TRAIL can significantly reduce the migration ability of tumor cells.

However, the role of TRAIL in platelets secreting TGF- β is not fully understood. We therefore investigated the role and mechanism of TRAIL in platelet-induced tumor metastasis in patients with colorectal cancer and in cultured colorectal cancer cells, in relation to the secretion of TGF- β 1.

Materials and methods

Tissue preparation

Tissues were obtained from patients undergoing elective colorectal resections between November 2015 and October 2016. All patients provided written informed consent and approval was obtained from the Medical Research Human Ethics Committee of the Fourth Affiliated Hospital of Hebei Medical University. Matched adjacent normal tissues >10 cm from the tumor edges were also obtained. Tissues were fixed in 4%

paraformaldehyde and embedded in optimum cutting temperature compound or paraffin for immunohistochemical staining. Paraffin-embedded tissues from 90 patients with colorectal cancer were chosen according to the original pathological diagnosis. Patients were re-diagnosed based on hematoxylin and eosin-stained sections by two experienced pathologists who were blinded to the source of the tissues, according to the American Joint Committee on Cancer Staging and Union for International Cancer Control classification guidelines.

Experimental animals

C57BL6 mice (9 to 12 weeks old, $n=5$) were maintained under specific pathogen-free conditions, fed with standard laboratory chow (Harlan Teklad, Indianapolis, IN, USA), and kept under a 12-hour light/dark cycle in the Animal Resource Core Facility of Case Hebei Medical University. All protocols were approved by the Institutional Animal Care and Use Committee of Hebei Medical University. Mice were injected intraperitoneally with TRAIL (1 $\mu\text{g/g}$) or phosphate-buffered saline (PBS; control) for 2 hour, and the distal tail was then cut off. Bleeding time was recorded and the amount of bleeding was measured using an ultraviolet spectrophotometer (SpectraMax i3x; Molecular Devices, Sunnyvale, CA, USA).

Preparation of human platelets

Blood was collected from healthy volunteers who had not taken any drugs known to affect platelet function for at least 14 days prior to the study. The samples were placed in 3.2% (w/v) trisodium citrate and platelet-rich plasma was obtained by centrifuging the blood for 10 minutes at $200 \times g$. Platelet suspensions were prepared as described previously.¹⁵ Platelet concentration was determined using a particle counter

(WS-Z1DUALPC; Beckman Coulter, Inc., Brea, CA, USA). Platelets were resuspended in plasma containing 5% dimethylsulfoxide for tumor cell invasion studies.

Tumor cell line culture

Two human colon cancer cell lines, HCT116 and HT29 adenocarcinoma cells, were obtained from the Shanghai Institute of Life Sciences, China. The cell lines were cultured as monolayers in McCoy's 5A medium (SH30200.01, HyClone Laboratories Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin in an atmosphere of 5% CO_2 in a cell holder at 37°C. Cells were subcultured three times each week.

Immunohistochemistry

Sections were blocked with 0.3% hydrogen peroxide, followed by preincubation with 5% normal goat serum and incubation with primary antibodies against CD62P (5 $\mu\text{g/mL}$, BD-553743, BD Biosciences, San Jose, CA, USA) at 4°C overnight. The sections were then incubated with biotinylated secondary antibody, followed by streptavidin-horseradish peroxidase and diaminobenzidine, and counterstained with hematoxylin. The staining intensity was determined by measuring the mean optical density at 595 nm by light microscopy using Image-Pro Plus (Media Cybernetics Inc., Bethesda, MD, USA).

Western blot

Platelets treated with TRAIL or PBS were washed and homogenized on ice using RIPA buffer (50 mM TRIS pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 1 mM phenylmethane-sulfonyl fluoride). Lysates containing equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto

polyvinylidene difluoride membranes using a semi-dry transfer system (Bio-Rad, Hercules, CA, USA). Blots were blocked with 5% nonfat dry milk in TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 2 hours at room temperature, and then probed with mouse monoclonal antibody against DR5 (1.5 $\mu\text{g}/\text{mL}$, ab16329, Abcam, Cambridge, MA, USA), mouse monoclonal antibody against DR4 (1.5 $\mu\text{g}/\text{mL}$, ab8414, Abcam), and rabbit polyclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:800; sc-32233; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The blots were then washed with TBST and incubated with fluorescence-conjugated goat anti-mouse/rabbit IgG (Rockland Immunochemicals Inc., Gilbertville, PA, USA) for 1 hour at room temperature. The immunoreactive bands were visualized using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA), and protein expression was quantified by densitometric analysis using Odyssey Imaging Software v3.0 (Li-Cor Biosciences).

Tumor cell invasion assay

The role of the TRAIL receptors DR5 and DR4 in platelet-induced invasion of HCT116 and HT29 cells was investigated by BD BioCoat Matrigel Invasion Chamber assay (BD Biosciences). Aliquots of 200 μL suspensions of 1×10^5 HCT116 and HT29 cells were seeded on the upper well, and 600 μL of serum-free culture medium was added to the lower chamber. Cell invasion was studied in the presence of platelets (1.5×10^8 platelets/mL) treated with and without anti-DR4 and anti-DR5 antibodies (1:50), TRAIL (1 $\mu\text{g}/\text{mL}$), or both. These treatments were added to the lower chamber of the invasion well for 40 hours. To assess the number of invaded cells, the filters were fixed with 4%

paraformaldehyde for 30 minutes at room temperature and stained with crystal violet. Each experiment was performed in duplicate and was repeated at least three times. The cells on the underside of the filter were counted under a microscope (Leica, Solms, Germany; 400 \times). Results were expressed as number of cells per field, corresponding to the average number of cells photographed for the corresponding treatment.

Vascular endothelial growth factor (VEGF) and TGF- β 1 measurement

Platelets were treated with ADP (10 μM) at room temperature for 2 minutes. The platelets were cultured by DMEM hyperglycemia medium. VEGF and TGF- β 1 release levels were then measured using the respective human or mouse enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions.

Flow cytometry analysis

Platelets were blocked with phycoerythrin-conjugated anti-DR4 antibody (3 $\mu\text{g}/\text{mL}$, Abcam) and fluorescein isothiocyanate-conjugated anti-DR5 antibody (3 $\mu\text{g}/\text{mL}$, Abcam), and incubated with fluorescein isothiocyanate-IgG. Five thousand platelets were acquired in a flow cytometer (Guava; Beckman Coulter, FL, USA) and analyzed using FCS Express software (De Novo Software, CA, USA).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from platelets by the TRIzol method (Pufei Biotechnology, Shanghai, China), and was reverse transcribed to cDNA. Real-time PCR was performed using a Light Cycler[®] 4800 System with specific primers for DR4, DR5, and GAPDH. The relative quantity values ($2^{-\Delta\Delta\text{Ct}}$, where Ct is the threshold cycle)

Table 1. Primer sequences of DR4, DR5, and GAPDH.

Name	Sequence	
DR4	For 5'-ATGGCGCCACCACCAGCTAGA-3'	Rev 5'-TGAGCAACGCAGACTCGCTGT-3'
DR5	For 5'-CAGGTGTGATTCAGGTGAAGT-3'	Rev 5'-GGACATGGCAGAGTCTGCAT-3'
GAPDH	For 5'-ACGGATTTGGTCGTATTGGG-3'	Rev 5'-CGTCTCTGGAAGATGGTGAT-3'

GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

of each sample were calculated, and are presented as fold change in gene expression relative to the control group. GAPDH was used as an endogenous control. The primer sequences of DR4, DR5, and GAPDH are given in Table 1.

Statistical analysis

Data are presented as the mean \pm standard error of the mean. A two-tailed χ^2 test was used to evaluate differences in expression between clinicopathological features and CD62P expression. Paired or unpaired data were compared by Student's *t*-tests. The data were analyzed using SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). For all statistical comparisons, $P < 0.05$ was considered statistically significant.

Compliance with ethical standards

All procedures were carried out in accordance with the ethical standards of the institutional ethical committee and with the 1964 Helsinki declaration and its later amendments. All applicable international, national, and/or institutional guidelines for the care and use of animals and humans were followed.

Results

TRAIL inhibits platelet-induced tumor cell invasion

CD62P expression was measured on activated platelets in human colorectal cancer

tissues. A total of 90 eligible patients with colorectal cancer met the selection criteria in The Cancer Genome Atlas database, including 50 men and 40 women (median age 58 years, range 28 to 82 years). CD62P expression levels were significantly higher in cancer tissues than in their paired adjacent normal mucosa ($P < 0.05$) (Figure 1a). When the patients were stratified according to CD62P expression level, platelet aggregation was significantly correlated with tumor stage ($P = 0.0024$), with greater platelet accumulation in stage T3 than in stage T2, and in T2 compared with T1. Platelet accumulation also differed among nodal stages ($P < 0.001$), with greater platelet accumulation in N2 than in N1, and in N3 compared with N2. Surprisingly, platelet aggregation was closely correlated with lymph node metastasis ($P < 0.001$) and vascular invasion ($P = 0.0158$), with significantly greater platelet accumulation in stage III/IV compared with stage I/II patients ($P < 0.001$) (Table 2). These results indicated that platelet accumulation in human colorectal cancer tissues was closely related to cancer progression.

We detected the effect of platelets on the invasion of tumor cells by transwell assay, and showed that the number of transmembrane cells was significantly increased in the platelet group compared with the control group ($P < 0.05$) (Figure 1b, c). These results suggest that platelets could promote tumor migration. The transwell assay results indicated that

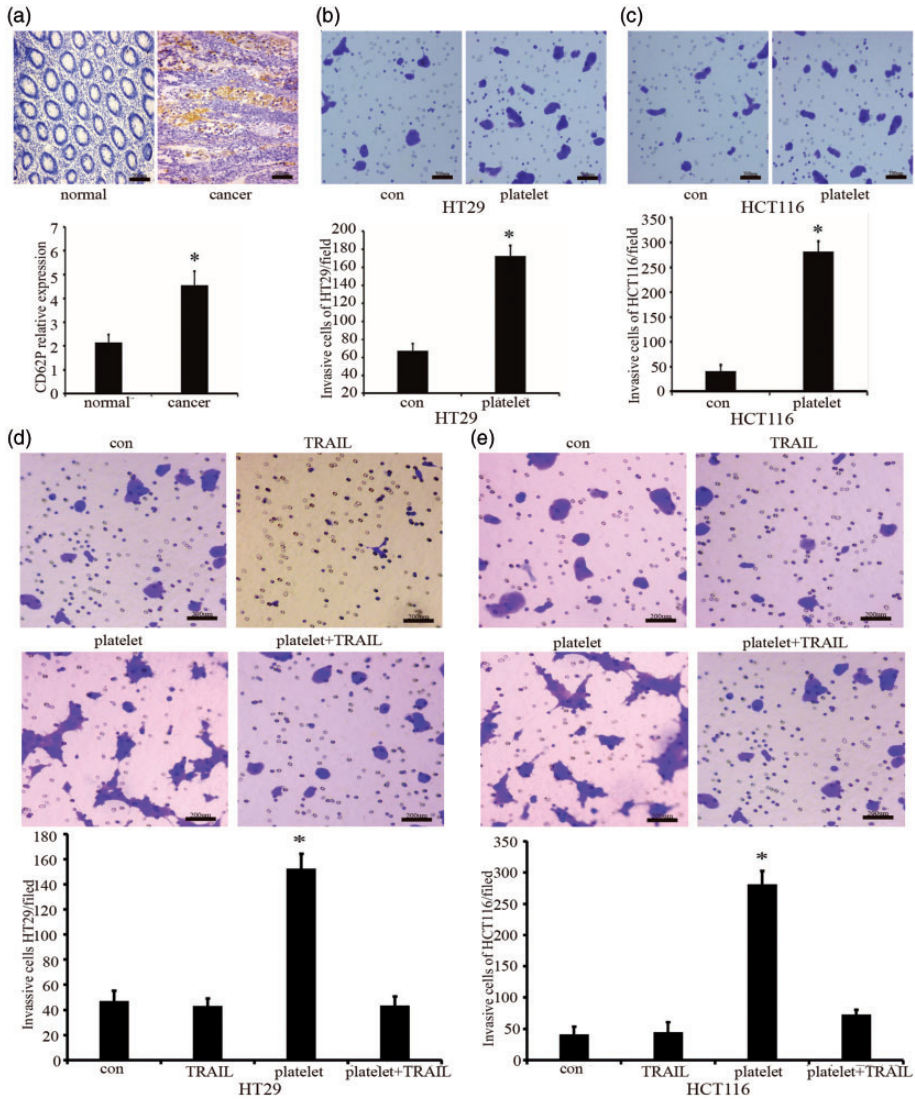


Figure 1. TRAIL inhibits platelet-induced tumor cell invasion. (a) CD62P levels in 90 sets colorectal cancer tissues and matched adjacent tissues determined by immunohistochemistry. (Hematoxylin stain). (b,c) Invasive abilities of HT29 and HCT116 cell lines cultured with or without platelets were determined by transwell assay. (Crystal violet stain). (d,e) Invasive abilities of HT29 and HCT116 cell lines treated with TRAIL, platelets, and TRAIL+platelets were determined by transwell assay. (Crystal violet stain). (n = 6 per group). Data are shown as mean ± standard error. *P < 0.05 compared with control group. Scale bars = 200 μm. con: control, TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

platelet treatment significantly increased the number of trans-membrane migrating HT29 and HCT116 cells compared with the control group, while treatment

with TRAIL plus platelets significantly reduced cell migration compared with cells treated with platelets alone (P < 0.05) (Figure 1d, e).

Table 2. Clinicopathological characteristics of 90 patients with colon cancer, stratified according to low or high CD62P protein expression.

Clinicopathological characteristic	Total n = 90 (%)	CD62P-low ^a n = 32 (35.56%)	CD62P-high ^a n = 58 (64.44%)	χ^2 value	P value
Sex					
Male	50 (55.56%)	19 (21.11%)	31 (34.44%)	0.2934	0.5881
Female	40 (44.44%)	13 (14.44%)	27 (30%)		
Age (years)					
≤60	47 (52.22%)	17 (18.89%)	30 (33.33%)	0.0162	0.8987
>60	43 (47.78%)	15 (16.67%)	28 (31.11%)		
Histological grade					
Well differentiated	7 (7.78%)	5 (5.56%)	2 (2.22%)	4.273	0.1181
Moderately differentiated	64 (71.11%)	21 (23.33%)	43 (47.78%)		
Poorly differentiated	19 (21.11%)	6 (6.67%)	13 (14.44%)		
Tumor stage					
T1	7 (7.78%)	5 (5.56%)	2 (2.22%)	14.39	0.0024*
T2	16 (17.78%)	10 (11.11%)	6 (6.67%)		
T3	53 (58.89%)	11 (12.22%)	42 (46.67%)		
T4	14 (15.56%)	6 (6.67%)	8 (8.89%)		
Nodal stage					
N1	39 (43.33%)	26 (28.89%)	13 (14.44%)	32.83	<0.0001*
N2	32 (35.56%)	5 (5.56%)	28 (31.11%)		
N3	18 (20%)	0	18 (20%)		
Lymph node invasion					
No	40 (44.44%)	29 (32.22%)	11 (12.22%)	36.93	<0.0001*
Yes	50 (55.56%)	5 (5.56%)	45 (49.44%)		
Vascular invasion					
No	76 (84.44%)	31 (35.56%)	45 (49.44%)	5.841	0.0158*
Yes	14 (15.56%)	1 (1.11%)	13 (14.44%)		
Clinical stage					
I/II	37 (41.11%)	26 (28.89%)	11 (12.22%)	33.04	<0.0001*
III/IV	53 (58.89%)	6 (6.67%)	47 (51.11%)		

P values were calculated by χ^2 test or independent t-tests for continuous data. *Significant difference.

^aExpression level of CD62P in normal humans (2×10^6 /mL) was used as the cut-off level to differentiate between low and high expression.

DR5 mediates the inhibitory effect of TRAIL on platelet-induced tumor cell invasion

We further investigated the expression of the TRAIL receptors DR4 and DR5 in platelets by flow cytometry, western blot, and real-time PCR. DR5 but not DR4 was expressed in platelets, as shown by flow cytometry (Figure 2a), and DR5 mRNA and protein expression in platelets

were demonstrated by real-time PCR (Figure 2b) and western blot (Figure 2c), respectively. We also examined the role of the receptor DR5 to clarify the mechanism by which TRAIL inhibited platelet-induced colorectal cancer metastasis. Platelets were pretreated with anti-DR4 and anti-DR5 antibodies before incubation with TRAIL and tumor cell invasion was measured by transwell assay, as described above. Both anti-DR5 antibody and TRAIL

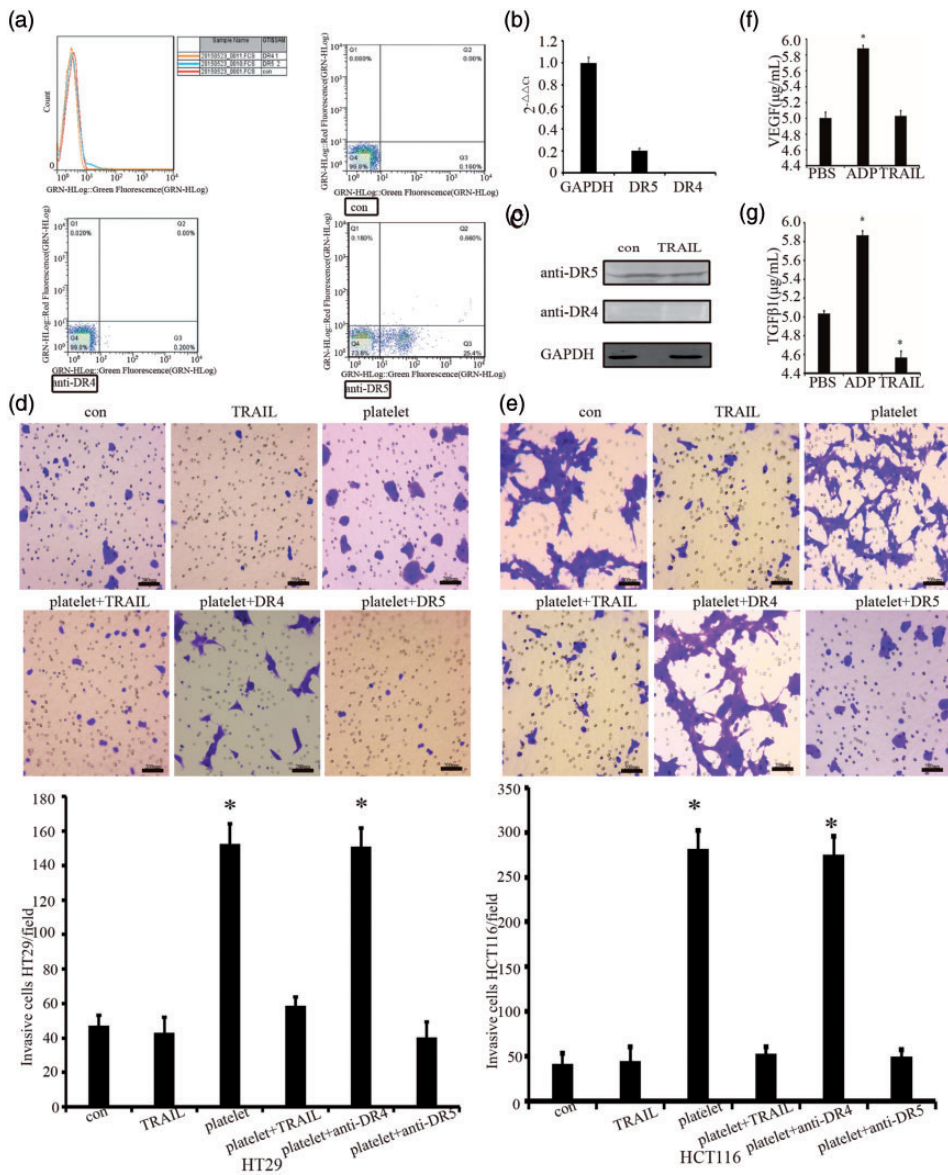


Figure 2. DR5 mediates the inhibitory effect of TRAIL on platelet-induced tumor cell invasion. (a,b) DR4 and DR5 expression were detected by flow cytometry and real-time polymerase chain reaction in platelets. (c) Detection of TRAIL receptors DR4/DR5 in platelets by western blot. (d,e) Invasive abilities of HT29 and HCT116 cell lines were determined by transwell assay in the TRAIL, platelet, platelet + TRAIL, platelet+DR4, and platelet+DR5 groups. (Crystal violet stain). * $P < 0.05$ compared with control group. Scale bars = 200 μ m. (f,g) VEGF and TGF- β 1 were detected by ELISA. Data are shown as mean \pm standard error. * $P < 0.05$ compared with PBS group. $n = 6$ per group. con: control, GAPDH, glyceraldehyde 3-phosphate dehydrogenase, TRAIL: tumor necrosis factor-related apoptosis-inducing ligand, PBS: phosphate-buffered saline.

significantly inhibited platelet-induced tumor cell invasion compared with the platelet group ($P < 0.05$) (Figure 2d, e). We also determined if TRAIL affected tumor cell metastasis by promoting TGF- β 1 secretion from activated platelets, and showed that VEGF and TGF- β 1 levels in the medium were increased in ADP-treated platelets compared with the control group. However, TGF- β 1 secretion was significantly reduced in TRAIL-treated platelets ($P < 0.05$) (Figure 2f, g).

TRAIL has no effect on circulating blood platelets in mice

In view of the advantages of TRAIL in tumor treatment, we determined if TRAIL affected bleeding *in vivo*. There was no significant difference in bleeding time or blood loss between mice treated with TRAIL and PBS ($P > 0.05$) (Figure 3), indicating that TRAIL did not affect bleeding *in vivo*.

Discussion

Thrombocytosis has recently been associated with prognosis in patients with a variety of cancers.¹⁶ Studies found that recombinant TRAIL protein inhibited the growth of tumor cells and even caused tumor regression, with no obvious damage to the host, suggesting potential applications for the treatment of tumors. In this study, we

investigated the inhibitory effect of promoting platelet apoptosis on the metastasis and invasion of colon cancer cells. The results unexpectedly showed that TRAIL prevented cancer cell metastasis and invasion by promoting platelet apoptosis and reducing the release of TGF- β 1.

We verified that platelets promoted tumor metastasis by immunohistochemistry and invasion assays, consistent with the results of previous studies.¹⁷ Platelets have been shown to promote tumor cell growth^{18,19} and play an important role in tumor metastasis. TRAIL has five types of receptors, of which DR4 and DR5 play prerequisite roles in signal transduction.^{20–23} However, the expression of DR4/DR5 by platelets remains controversial. We conducted transwell experiments and demonstrated that TRAIL inhibited platelet-induced invasion of colorectal cancer cells. We also detected platelet expression of DR4/DR5 by flow cytometry, real-time PCR, and western blot, all of which showed that platelets expressed TRAIL receptor DR5, but not DR4. Transwell assays further showed that TRAIL inhibited platelet-induced tumor metastasis via its surface receptor DR5.

The details of these mechanisms warrant further investigation. Previous studies have shown that platelets secrete many bioactive factors, including VEGF and TGF- β , both of which can facilitate tumor metastasis.¹⁹ We accordingly detected TGF- β 1 and VEGF levels in the supernatant from platelets incubated with TRAIL, and showed that TRAIL inhibited platelet secretion of TGF- β 1, but not VEGF. Previous research reported that the epithelial–mesenchymal transition was a significant mechanism of tumor invasion and metastasis, and that TGF- β 1 was a key initial step in tumor metastasis, while epithelial–mesenchymal transition was mainly caused by the TGF- β 1/Smad3 signaling pathway, which can promote tumor metastasis.²⁴

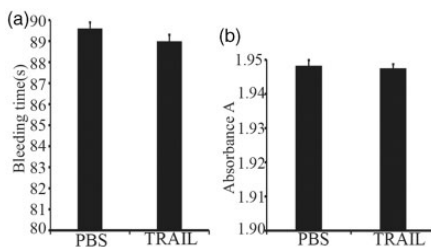


Figure 3. TRAIL has no effect on circulating blood platelets in mice. PBS: phosphate-buffered saline, TRAIL: tumor necrosis factor-related apoptosis-inducing ligand. Absorbance measured at 575 nm.

Given the role of TRAIL in local platelets, we also investigated its role in the systemic blood circulation in mice *in vivo*, and showed that TRAIL had no effect on bleeding time or bleeding volume, and thus did not affect the body's blood circulation.

However, this study also had some limitations. First, the number of human samples was relatively small. Furthermore, additional knockout or overexpression experiments should be conducted to verify the function of TRAIL.

In conclusion, this functional study found that TRAIL could inhibit metastasis and invasion of colon cancer cells by promoting platelet apoptosis and reducing the release of TGF- β 1. These findings identify potential new drug targets for the inhibition of tumor metastasis. However, TRAIL has certain disadvantages as an anti-cancer agent, including the existence of liver toxicity and the fact that many tumor cell lines are resistant to TRAIL. Further studies are therefore needed to clarify the mechanisms responsible for TRAIL resistance and to develop strategies to overcome this resistance, as well as establishing methods for achieving high efficiency tumor cell killing with low toxicity.

Acknowledgements

We are thankful to all respondents of this study and workers from the long-term care facilities for their cooperation.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This work was supported by National Natural Science Foundation of China grants 81372150 to Bing-Hui Li and 91439114, 31471092, 31271222, 91739301, and 91849102 to Mei Han.

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