ARTICLE

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Delivery of Apoptotic Signal to Rolling Cancer Cells: A Novel Biomimetic Technique Using Immobilized TRAIL and E-Selectin

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ABSTRACT: The survival rate for patients with metastases versus localized cancer is dramatically reduced, with most deaths being associated with the formation of secondary tumors. Circulating cancer cells interact with the endothelial lining of the vasculature via a series of adhesive interactions that facilitate tethering and firm adhesion of cancer cells in the initial steps of metastasis. TNF-related apoptosis-inducing ligand (TRAIL) holds promise as a tumor-specific cancer therapeutic, by inducing a death signal by apoptosis via the caspase pathway. In this study, we exploit this phenomenon to deliver a receptor-mediated apoptosis signal to leukemic cells adhesively rolling along a TRAIL and selectin-bearing surface. Results show that cancer cells exhibit selectin-mediated rolling in capillary flow chambers, and that the rolling velocities can be controlled by varying the selectin and selectin surface density and the applied shear stress. It was determined that a 1 h rolling exposure to a functionalized TRAIL and E-selectin surface was sufficient to kill 30% of captured cells compared to static conditions in which 4 h exposure was necessary to kill 30% of the cells. Thus, we conclude that rolling delivery is more effective than static exposure to a TRAIL immobilized surface. We have also verified that there is no significant effect of TRAIL on hematopoietic stem cells and other normal blood cells. This represents the first demonstration of a novel biomimetic method to capture metastatic cells from circulation and deliver an apoptotic signal.

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KEYWORDS: apoptosis; cancer; metastasis; TRAIL; E-selectin; rolling; cell adhesion

Introduction

Tumor metastasis consists of a series of discrete steps that move the tumor cells from the primary site to a distal location. The tumor cells must invade the surrounding tissue and enter either the bloodstream or the lymphatic system, survive the circulation, and adhesively interact with the vasculature that facilitates tethering, rolling, and eventually firm adhesion or arrest. Following arrest, the tumor cells extravasate into the distal site and grow into a secondary tumor (Chambers et al., 1995, 2000, 2001; MacDonald et al., 2002). Although surgery, radiation therapy, and chemotherapy are effective in controlling many cancers at the primary site, the development of metastatic cancer signals a poor prognosis. The survival rate for patients with such metastatic cancers is dramatically reduced, with most deaths being associated with the formation of secondary tumors. This makes metastatic tumors an important factor in determining the outcome of the disease.

Tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) or Apo2L is a 40 kDa, type II transmembrane protein identified on the basis of sequence homology to FasL and TNF. TRAIL is known to bind five known receptors: two of which are death receptors (DR4 and DR5) that signal apoptosis while the other three are decoy receptors (DcR1, DcR2, and DcR3) that do not signal apoptosis due to a shorter, non-functional cytoplasmic tail (Plasilova et al., 2002). Unlike other members of the TNF family, TRAIL exerts its cytotoxic effects on malignant cells without any adverse effects on most non-cancerous cells (Ashkenazi, 2002; Fricker, 1999; Held and Schulze-Osthoff, 2001; Plasilova et al., 2002) making TRAIL a promising therapeutic agent for cancer treatment. Recombinant soluble human TRAIL has been shown to induce apoptosis in several cancer cell lines and mouse xenografts (Ashkenazi et al., 1999; Gazitt, 1999; Mitsiades et al., 2001; Oikonomou

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et al., 2007; Walczak et al., 1999; Yu et al., 2000). Despite the broad range and specificity of TRAIL to induce apoptosis in cancerous cells, TRAIL has been found to induce apoptosis in non-cancerous human cells from the liver, brain, and keratinocytes (Jo et al., 2000; Leverkus et al., 2000; Nitsch et al., 2000). Besides these documented side effects, Zamai et al. (2000) have suggested that TRAIL may alter erythropoiesis. All these observations argue against systemic delivery of TRAIL. A delivery system where highly localized concentrations of TRAIL may be given to cancer cells without releasing TRAIL systemically is strongly warranted. Existing devices that filter blood for cancerous cells have been associated with patient discomfort. Moreover, these devices do not filter blood on a continuous basis. This may lead to a buildup of tumor forming cancer cells between treatments (Edelman et al., 1996; Fruhauf et al., 2001; Perseghin et al., 1997).

In this study, we demonstrate a novel biomimetic method to adhesively capture metastatic cancer cells from the peripheral circulation, expose the captured cells to a high concentration of TRAIL and induce an apoptotic signal, thus neutralizing the cancer cell (Fig. 1A).

Materials and Methods

Reagents and Antibodies

Human serum albumin (HSA), bovine serum albumin (BSA), HEPES, EDTA, and $CaCO_3$ were all obtained from Sigma–Aldrich (St. Louis, MO). RPMI 1640 cell culture media, fetal bovine serum (FBS), 1× trypsin, penicillin–streptomycin (PenStrep), Hank's balanced salt solution (HBSS), phosphate buffered saline (PBS), and Dynal® CD34+ Progenitor Cell Isolation System were all obtained from Invitrogen (Grand Island, NY). His-tagged recombinant human TRAIL, human methylcellulose complete media, recombinant human E-selectin-IgG chimera, and TACS Annexin-V FITC Apoptosis Detection kit were purchased from R&D Systems (Minneapolis, MN).

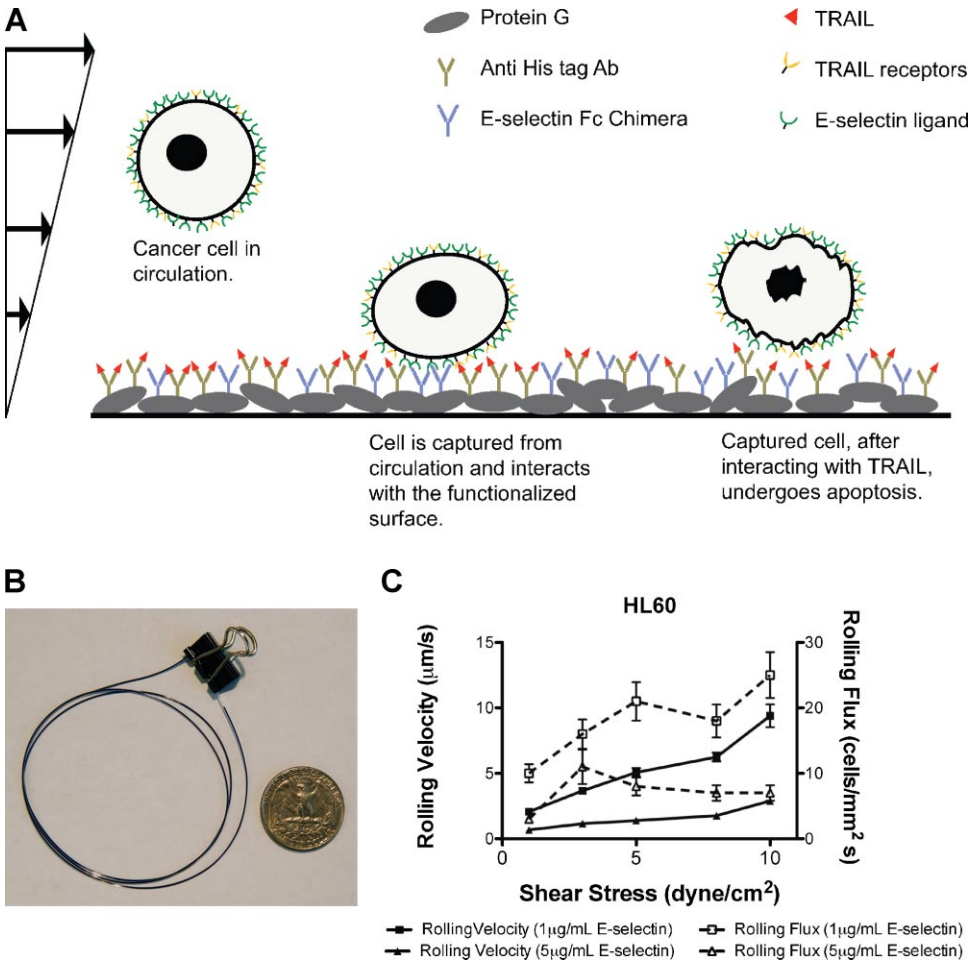

Figure 1. **A**: Schematic of the two-receptor system for capture of cancer cells from peripheral circulation and induction of apoptosis signal to the captured cells. **B**: Prototype capillary flow chamber which is functionalized with TRAIL and E-selectin. This tube has been filled with dye to aid viewing. **C**: Rolling velocity and rolling flux of HL60 cells for two different concentrations of E-selectin as a function of shear stress. The data plotted is average ± SEM ($n = 3$).

Protein-G and anti-His tag antibody were purchased from EMD Biosciences (San Diego, CA). Ficoll-Paque™ PLUS was obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Mouse anti-human TRAIL—PE and Mouse anti-human CD62E—FITC antibody were purchased from Abcam, Inc. (Cambridge, MA). Mouse anti-human TRAIL DcR1, DcR2, DcR3, DR4, and DR5, all conjugated to PE, were purchased from Biolegend (San Diego, CA). Quantum simply cellular (QSC) beads were purchased from Bangs Laboratories, Inc. (Fisher, IN).

Cell Lines and Cell Culture

Acute myeloid leukemic (AML) cell line, HL60 (ATCC number CCL-240), KG1a (ATCC number CCL-264.1), and Colon cancer cell line Colo205 (ATCC number CCL-222) were obtained from ATCC (Manassas, VA). Two prostate cancer cell lines DU145 and PC3 were obtained from Prof. Yi Fen-Lee (University of Rochester). These cell lines were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% (v/v) FBS, and 100 U/mL PenStrep (complete media) under humidified conditions at 37°C and 5% CO_2. Cells in suspension (KG1a and HL60) were cultured such that the cell count did not exceed 1×10^6 cells/mL while the adherent cells were maintained such that 90% confluence was not exceeded.

Cell Preparation for Rolling Experiments

Adherent cell lines (Colo205, DU145, and PC3) were mildly trypsinized for 5 min, following which the cells were allowed to sit in complete media, 5% CO_2 and humidified conditions for up to 5 h before use to ensure re-expression of surface receptors. All cells (including HL60 and KG1a) were washed twice with 1× PBS at 1,000 rpm in Allegra X-22 refrigerated centrifuge at 4°C and resuspended in the flow buffer at a concentration of 1×10^6 cells/mL. The flow buffer consisted of HBSS without Ca^{2+} and Mg^{2+} supplemented with 0.5% (w/v) HSA, 10 mM HEPES, and 2 mM $CaCO_3$. For rolling experiments, at least 90% viability of cells was confirmed by trypan blue exclusion dye.

Preparation of Immobilized Protein Surfaces

Recombinant human E-selectin-IgG was dissolved in PBS to a final concentration of 100 μg/mL. In addition, stock solutions of TRAIL, protein-G and anti-His tag antibody in PBS were prepared at 20, 100, and 200 μg/mL, respectively. Aliquots of the stock solutions were stored at −20°C and used as needed within 60 days. The surface was first incubated with 10 μg/mL protein-G solution for 1.5 h, followed by a 2 h incubation with selectin chimera (0.1–5 μg/mL) or a combination of selectin chimera and anti-His tag antibody (5 and 10 μg/mL, respectively), and then the surface was incubated with 2–20 μg/mL TRAIL solution. Each incubation step was followed with three washes with PBS. All incubations were conducted at room temperature and successful immobilization was confirmed by immunofluorescence.

Mononuclear Cell (MNC) and CD34+ Hematopoietic Stem and Progenitor Cell (HSPC) Isolation

All human subject protocols have been approved by the Research Subjects Review Board (RSRB) of the University of Rochester. Adult bone marrow (ABM) and peripheral human blood were collected from healthy willing donors after informed consent into vacutainer tubes containing heparin and allowed to equilibrate at room temperature before use. Ten to fifteen milliliters of ABM was diluted with PBS to a final volume of 35 mL, then carefully layered over 10 mL Ficoll-Paque PLUS and centrifuged at 1,600 rpm for 30 min. MNCs were collected and washed twice with PBS before being resuspended in flow buffer to the desired final concentration.

The Dynal® beads cell selection system was used for isolation of CD34+ HSPCs as per manufacturer's instructions. Briefly, anti-human CD34 antibody conjugated to 4.5-μm diameter paramagnetic beads was added to MNC suspension at 4×10^7 to 4×10^8 cells/mL and allowed to incubate at 4°C for 30 min before being placed in a magnetic field. The paramagnetic beads, attached to CD34+ cells, precipitated within the magnetic field so that the unselected CD34− cells could be removed and collected. The CD34+ cells were released from the antibody–bead complex using a competitive peptide, washed and resuspended in HBSS+ to the desired final concentration for experimentation. Mean CD34+ cell purity used for experiments was about 70% as determined by flow cytometry.

Colony Forming Assay

MNCs (5×10^4 cells/mL) were suspended in 3 mL of human methylcellulose complete media. The media purchased contained preadded recombinant human SCF (50 ng/mL), recombinant human GM-CSF (10 ng/mL), recombinant human IL-3 (10 ng/mL), and recombinant human Epo (3 U/mL). Soluble TRAIL was added at a concentration of 2 μg/mL and mixed to ensure uniform distribution of cells and TRAIL. After 14 days, the colonies were counted. All experiments were performed in duplicate.

Receptor Surface Expression

The average number of death and decoy receptors on cancer cells was determined by flow cytometry calibration with QSC beads. Briefly, beads were incubated for 45 min with a PE conjugated antibody specific to the antigen on the beads. Beads with different numbers of antibody binding sites were mixed and run through a flow cytometer. Populations of

beads corresponding to different numbers of antibody binding sites yield progressively increasing peaks in the fluorescence channel corresponding to the number of antibody binding sites. Median value of each peak was obtained using FlowJo v8.7.1. Using these median fluorescent channel values and the number of antibody binding sites (reported by the manufacturer), a calibration curve was generated using QuickCal v2.3 (Bangs Labs, Fishers, IN).

Following the calibration step, cancer cell lines were incubated with PE conjugated anti-human DcR1, DcR2, DcR3, DR4, and DR5 on rotating platform for 45 min at room temperature. These cells were then analyzed on a Guava EasyCyte Mini flow cytometer. The peak fluorescence channel was recorded for each receptor. From the calibration curve, the peak was converted to the number of death and decoy receptors. Three separate measurements were performed for each cell type and receptor.

Static Experiments

Cancer cell lines were cultured in complete media with soluble TRAIL (0–2 μg/mL) in a 24 well plate for 48 h. In a similar fashion, the effect of TRAIL on ABM was studied by culturing ABM with TRAIL at a concentration of 2 μg/mL. In some experiments, multiwell plates were functionalized with proteins as described earlier. Cells were then cultured on the functionalized surface in complete media, 37°C, 5% CO_2, and humidified conditions for up to 48 h.

Rolling Experiments

A 50-cm long, 300 μm internal diameter Micro-Renathane tubing (Fig. 1B) was obtained from Braintree Scientific (Braintree, MA), and secured to the stage of the Olympus IX81 motorized inverted research microscope (Olympus America, Inc, Melville, NY). The microscope was equipped with a CCD camera (model no: KP-M1AN, Hitachi, Tokyo, Japan) connected to either a S-VHS videocassette recorder (model no: SVO-9500MD2, Sony Electronics, Park Ridge, NJ) or a DVD recorder (model no: DVO-1000MD, Sony Electronics) to facilitate image capture for offline analysis. A syringe pump (KDS 230, IITC Life Science, Woodland Hills, CA) was used to control the flow rate of the cell suspension. Cells were loaded on the surface at a shear stress of 0.5–1 dyne/cm^2 for 3 min following which the flow experiment was performed. Flow experiments on functionalized capillary flow chamber surfaces were performed at 2.5 dynes/cm^2, for a period of 1 h. At the end of 1 h, cells were categorized into two fractions, "cells on surface"—cells that were rolling on or remained on the surface at the end of the experimentation period and "cells in flow"—cells that were collected in the syringe.

The cells on the surface were harvested using 5 mM EDTA and air embolism at 10 dyne/cm^2. These cells were then either seeded at 100,000 cells/mL and then counted at day 4, or cultured and analyzed by Annexin-V assay. E-Selectin and His-tag antibody (without TRAIL) functionalized surfaces were used as negative controls.

Data Analysis

"Rolling" cells were defined as those observed to translate in the direction of flow with an average velocity less than 50% of the calculated hydrodynamic free stream velocity. Rolling flux was determined by counting the number of cells crossing a line drawn in the field of view perpendicular to the flow direction, over a period of 1 min.

All cell experiments were analyzed by Annexin-V apoptosis assay on a BD FACSCaliber flow cytometer or the Guava EasyCyte Mini. Instructions provided by the manufacturer were followed to prepare samples for flow cytometry. Briefly, Annexin-V is an anticoagulant that binds to negatively charged phospholipids. Flipping of phosphatidylserine (PS) in the cell membrane to the extracellular side (enabling Annexin-V to bind to PS) is considered an early event in apoptosis and is considered important for macrophage recognition of apoptotic cells. Cells that label for propidium iodide (PI), a DNA stain, possess a compromised cell membrane. Based on the dye taken up by cells, they are classified into four categories: viable cells (negative for Annexin-V and PI), early apoptotic cells (positive for Annexin-V only), late apoptotic cells (positive for Annexin-V and PI), and necrotic cells (positive for PI only).

Where appropriate, the Student's *t*-test was employed at significance level of $\alpha = 0.05$. All statistical analyses were performed using GraphPad Prism 5.0a for Mac OS X (GraphPad Software, San Diego, CA, www.graphpad.com).

Results

Cancer Cells Exhibit a Shear Dependent Rolling on Functionalized E-Selectin

HL60 cells were rolled in a capillary tube 50 cm long and 300 μm internal diameter functionalized with E-selectin/Fc chimera at different shear stresses ranging from 1 to 10 dynes/cm^2. The surface density of E-selectin molecule was varied by incubating the tube with different concentrations of E-selectin solution at room temperature. Cells were suspended in flow buffer such that the concentration of cells was 1×10^6 cells/mL and were loaded on the surface at 0.5 dyne/cm^2. Shear stress was applied to the cells by flowing fluid and recorded for offline analysis. The average rolling velocity for each experiment was computed and the rolling flux was determined. Rolling was observed at 1 and 5 μg/mL incubation concentrations of E-selectin. No adhesive interaction was observed with 0.1 μg/mL E-selectin concentration while extremely slow rolling was observed at 5 μg/mL of E-selectin. At higher concentrations of

E-selectin (10 μg/mL E-selectin and higher) distinguishing firmly adherent and rolling cells became difficult. From these experiments we found that 5 μg/mL of E-selectin gave stable rolling with relatively slow rolling velocities. HL60 cell line shows a shear dependence of rolling velocity and rolling flux (Fig. 1C). By varying the E-selectin site density we were able to achieve different rolling velocities at a given shear stress. These results were used determine a suitable E-selectin density (5 μg/mL) to produce physiological rolling velocities at a representative wall shear stress.

Cells Show Different Sensitivity to Soluble TRAIL

With reports of TRAIL-resistant cancer cells (Bouralexis et al., 2003; Ng and Bonavida, 2002; Zisman et al., 2001), we investigated the effect of soluble TRAIL on our model cell lines HL60, KG1a, Colo205, DU145, and PC3. Cells at a concentration of 300,000–500,000 cells/mL were cultured in complete media at 37°C, 5% CO_2, and humidified conditions with varying concentrations (0–2 μg/mL) of TRAIL for a period of 48 h. These cells were then collected and examined for viability by Annexin-V assay (Fig. 2A–E). Since the primary objective of the work presented here is to neutralize cancer cells, irrespective of the mode of death, we compare viable cells in our treated and untreated samples.

In general, all cell lines showed a decrease in viability when treated with TRAIL. The cell lines Colo205 and HL60 were the most sensitive to TRAIL while KG1a and DU145 showed the most resistance. The PC3 cell line had sensitivity intermediate of HL60 and DU145. Among the leukemic cell lines, KG1a was more resistant to TRAIL than HL60. Even at 1.8 μg/mL about 70% of KG1a were viable, while the HL60 reached a plateau in viability at 0.1 μg/mL. Viability

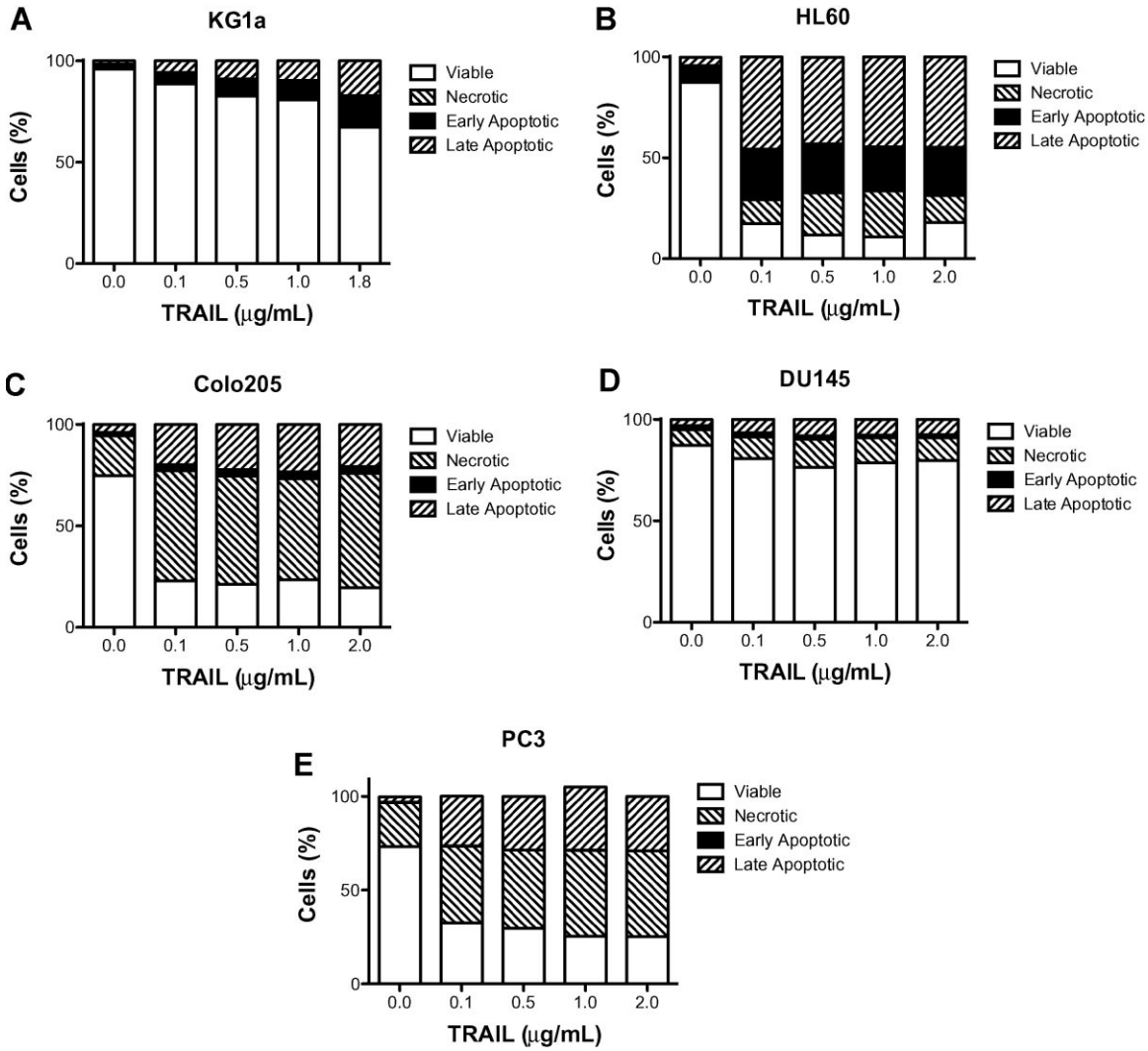

Figure 2. Dose response to soluble TRAIL for **A**: KG1a, **B**: HL60, **C**: Colo205, **D**: DU145, and **E**: PC3 after 48 h. KG1a and HL60 are AML cell lines, Colo205 is a colon cancer cell line and DU145 and PC3 are prostate cancer cell lines. Stacked bars indicate viable, early apoptotic, late apoptotic, and necrotic fractions.

observed at 0.1 μg/mL was around 15–20%. Increasing the TRAIL dosage beyond 0.1 μg/mL did not decrease the viability significantly, while a further decrease in viability of KG1a may be possible at higher dosages of TRAIL.

Colo205 showed a plateau similar to that of HL60 at 0.1 μg/mL with around 15% viable cells. The prostate cancer cell lines PC3 and DU145 seemed to be relatively more resistant to TRAIL and showed a plateau at 0.1 μg/mL of TRAIL. Of the two prostate cancer cell lines, DU145 was more resistance to soluble TRAIL while PC3 cells showed resistance intermediate to that of KG1a and DU145 cell lines.

In a separate set of experiments, the cells were incubated with PE-conjugated antibody to the death and decoy receptors present on the cell membrane to quantify the number of receptors present. We did not see any significant correlation between the numbers of death and decoy receptors and the sensitivity of cancer cells toward soluble TRAIL. For instance, cell lines that were most sensitive to TRAIL expressed comparable total numbers of death and decoy receptors, while the more resistant cell lines, DU145, had more death receptors than decoy receptors. The average receptors counts from three independent experiments are summarized in Table I. Values represented are average ± SD.

Soluble TRAIL has no Significant Effect on Adult Bone Marrow Cells

Zamai et al. (2000) have suggested that TRAIL may affect erythropoiesis. ABM cells, isolated as described in the Materials and Methods Section, were treated with 2 μg/mL of soluble TRAIL and cultured at 37°C and humidified conditions for 48 h. These cells were then collected and analyzed by Annexin-V assay for viability. Treating the ABM cells with this high dose of TRAIL (median lethal dose, 50% or LD_{50} ~12 ng) had negligible effect on the viability of both CD34+ and CD34− bone marrow cells (Fig. 3A,B). Plasilova et al. (2002) have reported that His-tagged TRAIL reduced the number of myeloid colonies (CFU-GM) but had no adverse effects on ABM stem cells.

To ascertain that CD34+ stem cells still maintain their function, a colony-forming assay was performed. We found that there was no statistical difference in the number of colonies formed in both the treated and the untreated samples, however a small decrease in the number of CFU-GMs with a small increase in BFU-Es of the treated samples was observed (Fig. 3C,D).

Immobilized TRAIL and Selectin Surface Produces Significant Killing of HL60 Cells

Tissue culture grade polystyrene surfaces were functionalized with TRAIL and selectin to test the efficacy of the proposed system under static conditions. A high rate of apoptosis for HL60 cells was observed, while the more TRAIL resistant, KG1a cells showed no significant difference (Fig. 4A,B). Cells at a concentration of $2.5–3.0 \times 10^5$ cells/mL were seeded in each experiment and cultured at 37°C and 5% CO_2 under humidified conditions. Similar experiments with surfaces functionalized with TRAIL alone and selectin alone were also performed at two different incubation concentrations of TRAIL. When incubated on these surfaces, HL60 cells demonstrated a dose-dependent rate of cell death (Fig. 4C). No significant effect on viability was observed in the case of E-selectin and His-tag Ab alone (data not shown).

In a separate experiment, the effect of static contact time of cells with a surface functionalized with TRAIL and E-selectin for 1 and 4 h (Fig. 5B) was studied. No significant kill was observed after 1 h static exposure to TRAIL and E-selectin immobilized surface, while a 28% decrease in viability was observed after 4 h static exposure. No significant kill was observed in both, 1 and 4 h static exposure to surfaces lacking the TRAIL molecule.

Flow Over Combined Surface for 1-h Kills About 30% of HL60 Cells Without Significant Effects on MNCs

HL60 cells were washed in 1× PBS and resuspended in flow buffer at a concentration of 1×10^6 cells/mL. These cells were loaded onto the functionalized surfaces at wall shear stress of 0.5–1 dynes/cm^2 for about 3 min after which the shear stress was increased to 2.5 dynes/cm^2. The loading step promotes initial cell contact with the surface. Cells were perfused in a capillary tube (300 μm internal diameter and 50 cm length) functionalized with proteins for 1 h. On average, about 10% of the total perfused cells interact adhesively with the surface and saturated the surface within a few minutes. At the end of 1 h, the cells present on the surface of each capillary flow chamber were collected using

Table I. Death and decoy receptor expression on KG1a, HL60, Colo205, DU145, and PC3.

	HL60 ($n=3$)	KG1a ($n=3$)	Colo205 ($n=3$)	DU145 ($n=4$)	PC3 ($n=3$)
DcR1	10,000 ± 740	15,510 ± 60	4,880 ± 70	4,730 ± 260	4,400 ± 160
DcR2	7,690 ± 210	9,830 ± 120	6,810 ± 240	6,350 ± 1,360	2,450 ± 290
DcR3	25,900 ± 270	25,380 ± 260	3,090 ± 150	8,540 ± 510	4,260 ± 270
DR4	32,040 ± 750	48,730 ± 610	29,550 ± 600	30,060 ± 6,830	25,840 ± 590
DR5	7,610 ± 200	45,550 ± 230	154,520 ± 1,450	342,780 ± 74,700	118,890 ± 1,960

The results represent the average ± SD ($n=3$).

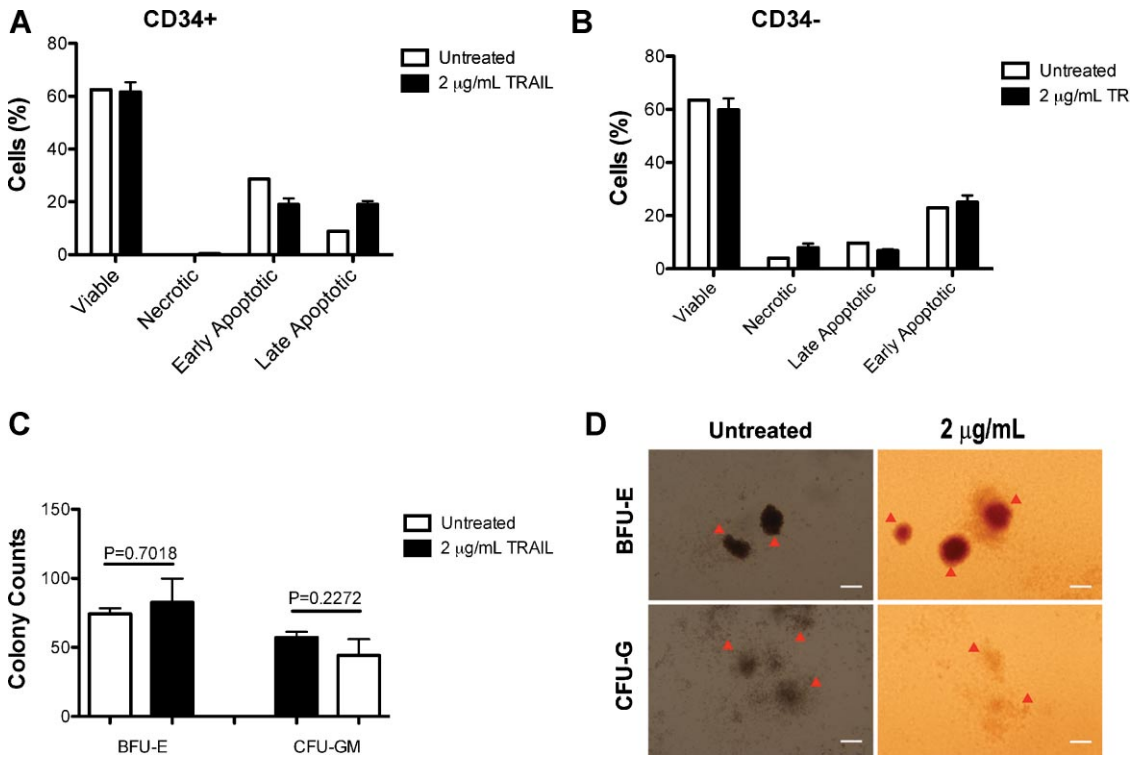

Figure 3. Viability and function of ABM. Cells were treated with 2 μg/mL of TRAIL in media and cultured for 48 h at 37°C, 5% CO_2, and humidified conditions. Dark bars represent the untreated cells while the lighter bars represent the treated cells. **A**: Viability CD34+ cells, **B**: viability of CD34− cells, **C**: colony counts for treated and untreated MNCs ($P > 0.05$), and **D**: BFU-E and CFU-GM as seen in untreated and the treated samples (scale bar = 100 μm). The values shown are average ± SEM ($n = 3$). Arrows indicate respective colonies.

5 mM EDTA and air embolism at 10 dynes/cm^2. The harvested cells were washed three times in PBS, counted and resuspended in RPMI complete media at 10^5 cells/mL and cultured either for four days and counted or cultured for 1 day and analyzed by Annexin-V assay for viability. The cells counts after 4 days are shown in Figure 5C. A 35% difference in cell number at day 4 was observed between the cells collected from the combined TRAIL and E-selectin surface and the control surface (E-selectin and His-tag Ab). Significance was determined by the Student's *t*-test and a $P < 0.001$ was determined. No significant apoptotic activity was seen immediately after the rolling experiment.

In the case where cells were cultured for 24 h followed by Annexin-V assay, a 30% difference in viable cells was seen between the cells harvested from the control, E-selectin and anti-His tag Ab, and the combined TRAIL and E-selectin surface (Fig. 5D). The E-selectin + His-tag Ab surface showed the same viability as the unrolled cells. Figure 5A is a representative plot of the original flow cytometry data. The results are divided into four quadrants with the lower left indicating viable cells, lower right indicating early apoptotic, upper right indicating late apoptotic, and upper left indicating necrotic cells.

To ascertain the effects of non-cancerous cells that may interact adhesively with the TRAIL and E-selectin surface, MNCs isolated from fresh human blood were allowed to flow over the functionalized microcapillary surfaces for 1 h at a wall shear stress 2.5 dyn/cm^2. Cells were then collected using air embolism and 5 mM EDTA, washed and cultured for 6 h followed by the Annexin-V assay. No significant difference in viability was observed in cells that rolled over the combined TRAIL and E-selectin surface and the E-selectin surface lacking TRAIL (Fig. 5E). Cells collected in the syringe at the end of the experiment had viability similar to the control cells suggesting little to no leaching of TRAIL from the surface (data not shown).

We did not observe any significant cell binding on surfaces immobilized with protein-G, protein-G and anti-His tag antibody, anti-His tag antibody only, His-tag antibody coupled TRAIL, and capillary flow chamber without any protein. The adherent cells harvested from these tubes were not sufficient in number to perform analysis (100–500 cells).

Discussion

In this study, we demonstrate for the first time, a novel biomimetic method to capture metastatic cancer cells from flow via adhesive interactions with selectins and induce an apoptotic signal. Our results suggest that by rolling cancer cells over the functionalized surface we are able to induce an

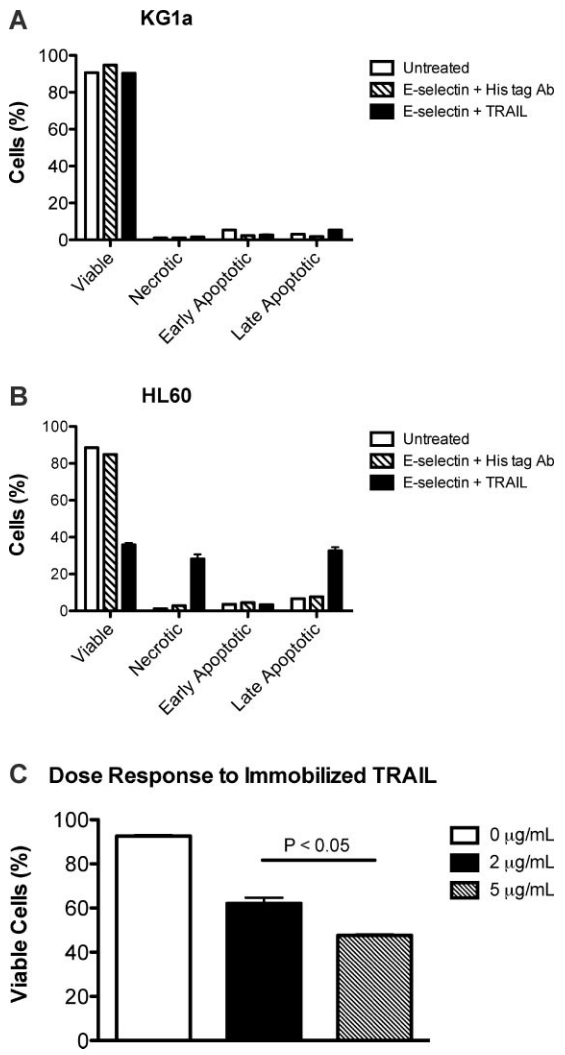

Figure 4. Effect of E-selectin and TRAIL on functionalized polystyrene surfaces. Surfaces were functionalized with 2.5 μg/mL E-selectin and 5 μg/mL of TRAIL. Cells were incubated at 37°C, 5% CO_2, and humidified conditions under static conditions for 48 h. The results shown are average ± SEM ($n = 3$). **A**: KG1a and **B**: HL60. **C**: Dose dependency of HL60 on TRAIL immobilized on a polystyrene surface. Surfaces were functionalized with 2 and 5 μg/mL of TRAIL and cultured under static conditions at 37°C, 5% CO_2, and humidified conditions for 44 h. The results are average ± SEM ($n = 3$). $P < 0.05$.

apoptotic signal to a greater population of targeted cells than cells under static conditions.

Cancer cells interact with the endothelial lining of the vasculature by a variety of adhesion molecules that facilitate tethering and arrest of blood borne cancer cells to the blood vessel wall as the initial step in metastatic tumor formation. The initial contact between the cancer cell and the vasculature is mediated by the selectin group (L-, P-, and E-selectin) of glycoproteins (Goetz et al., 1996; Kim et al., 1999; Orr et al., 2000; Orr and Wang, 2001; Waugh and Evans, 1979). This contact with the endothelium initiates a cascade of activation events, similar to that of neutrophil recruitment during inflammation, and ultimately leads to the development of a metastatic tumor (Orr et al., 2000).

Sipkins et al. (2005) have characterized bone marrow microdomains susceptible to tumor engraftment. Using immunofluroscence they mapped the spatial distribution of adhesion molecules on endothelial cells and the chemoattractant stromal-cell-derived factor 1 (SDF-1). Then using a leukemic cell line, they showed that cell homing and engraftment correlated with the spatial distribution of E-selectin and SDF-1. Cells that were inhibited from homing into bone marrow remained in peripheral circulation. This study suggests the potential for using our device to adhesively capture cancer cells in peripheral circulation to induce an apoptotic signal and hence prevent or reduce the metastatic load.

Devices that reduce the blood borne metastatic load have been proposed by several researchers (Edelman et al., 1996; Fruhauf et al., 2001; Perseghin et al., 1997). These devices require the passing of blood through a mechanical filter. Though these devices are efficient in filtering cancer cells from peripheral circulation, they have inherent limitations. Being external devices, these devices require catheterization, which increases patient discomfort and chances of secondary infection. In addition, filtration of cells is not on a continuous basis and consequently may lead to accumulation of potential tumor forming cancer cells in circulation between treatments. With our proposed device, we eliminate these problems by making the device implantable. This would allow screening of cancer cells on a continuous basis. The device may be customized by functionalizing the surface with specific chemoattractants, proteins and small peptides or molecules to target specific cell types, thus extending the utility of the device.

We have recently shown that using a P-selectin coated tube we are able to enrich CD34+ hematopoietic stem cells from peripheral circulation of G-CSF treated rats (Wojciechowski et al., 2008) and have shown that the same method can also be used to enrich CD34+ hematopoietic stem cells from human bone marrow (Charles et al., 2007; Narasipura et al., 2008). Cells collected in this manner may be used for stem cell transplants to leukemic patients. Unlike allogeneic transplants, wherein stem cells are collected from a different donor, autologous transplants, wherein cells are collected from the patient, do not have the complication of immune rejection although autologous transplants have an associated risk of reintroducing cancer cells. The proposed device may be used ex vivo to capture and neutralize cancer cells before autologous stem cell transplant to the patient. The device may also be used to filter and neutralize cancer stem cells that are believed to be tumorigenic in contrast to the bulk of cancerous cells that are non-tumorigenic (Huntly and Gilliland, 2005; Li et al., 2007; Park et al., 1971).

The receptor surface expression we have measured, do not show a correlation with sensitivity toward TRAIL. The more resistant cell lines such as KG1a and DU145 show a greater number of death receptors than decoy receptors. In general, the more resistant cell lines, DU145 and KG1a both express a

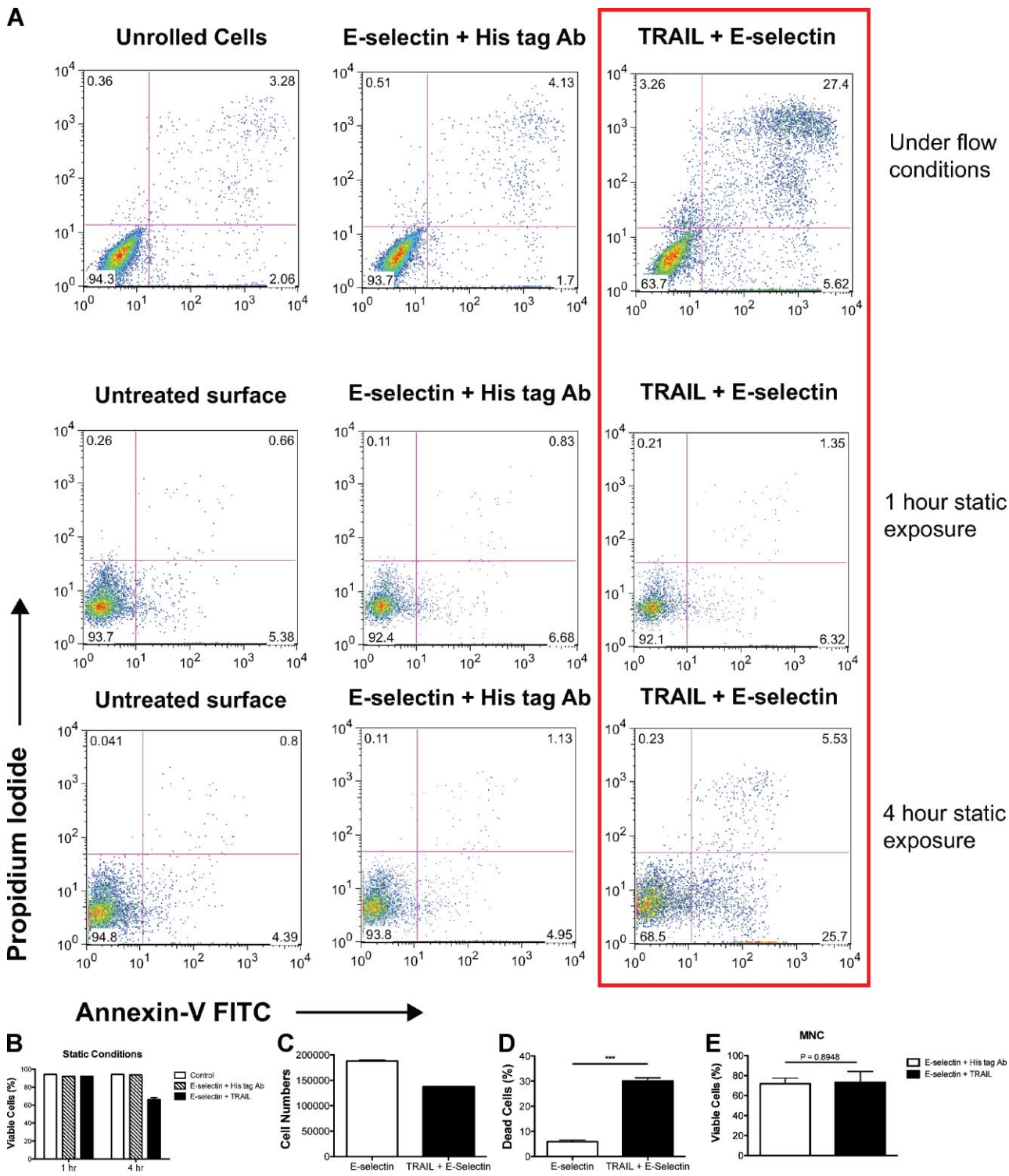

Figure 5. Effect of TRAIL and E-selectin functionalized surfaces. The surfaces were incubated with 10 μg/mL protein-G, 10 μg/mL of His-tag Ab, 5 μg/mL of E-selectin, and 20 μg/mL TRAIL as described earlier. **A:** Representative flow cytometry plots. **B:** Under static loading conditions for 1 and 4 h. Results are average ± SEM ($n = 2$). **C:** Cell counts at day 4 following 1 h of rolling on functionalized surface. Each experiment was performed in duplicate and the value plotted is the average ± SD ($n = 5$). $^{***}P < 0.001$. **D:** Percent dead cells (100 − % viable cells) after 1 h of rolling on the functionalized surface followed by 24 h incubation. An average difference of 30.08 ± 0.64% between E-selectin + His-tag Ab surface and the combined surface of TRAIL and E-selectin is observed. The results plotted are average ± SD ($n = 3$). $^{***}P < 0.001$. **E**: Percent viable MNCs after 1 h rolling on functionalized surfaces at a wall shear stress of 2.5 dyn/cm^2. Cells were harvested using air embolism and 5 mM EDTA, washed and cultured for 6 h. MNCs were isolated from freshly collected human blood. The results plotted are average ± SEM ($n = 3$), $P = 0.8948$.

higher number of death receptors while the more sensitive cell lines PC3 and HL60 express lower number of death receptors. These observations suggest that the ratio of death to decoy receptors may not play an important role in determining the sensitivity of cancer cells toward TRAIL and that there is some intrinsic mechanism that makes cells more resistant to TRAIL.

We have demonstrated that it is possible to kill about 30% of the rolling cells using our method with a very short exposure to TRAIL. Wojciechowski and Sarelius (2005) showed that in the case of leukocyte trafficking, only about 10% of the arrested cells manage to transmigrate through the endothelial lining. The remainder of activated leukocytes eventually re-enter the blood flow. If the experiments were conducted in a closed loop, this would allow multiple passes of cells over the surface and if conducted over longer periods of time, would yield a higher kill rate that may significantly reduce the metastatic load. From the available literature, we have identified various cancer cell lines that are sensitive to selectins and TRAIL. Cell lines showing reactivity toward both molecules are identified as potential targets for initial experimentation.

Recently, reports of chemotherapeutic drugs, when used in sub-lethal doses, sensitize many cancer cell lines to TRAIL. These drugs when used in combination with TRAIL are shown to have additive and super-additive effects on cancer cells (Cuello et al., 2001; Keane et al., 1999; Mizutani et al., 2001). The proposed device could be used in conjunction with other treatments, thereby increasing the efficacy of the combined treatment. Literature also suggests that cancer cells may acquire immunity toward chemotherapy or radiotherapy by limiting drug uptake through the cell membrane or developing a DNA repair mechanism. Resistant cells when treated with TRAIL, chemotherapeutic drug, or a combination of the two, were found to undergo apoptosis. The same principle may be applied to cells that become resistant to either chemotherapy by treating with TRAIL, thereby expanding the utility of the device.

Of particular interest is the fact that cells rolling over a surface functionalized with TRAIL and E-selectin for 1 h produces a kill rate comparable to cells sitting on a similar functionalized surface for about 4 h. It should be noted that the surfaces used in the two cases were different types of plastics, however the incubation concentrations of the proteins was the same. To obtain an estimate of the amount of TRAIL present on the two different plastic surfaces, the surfaces were incubated with fluorescently labeled antibody against TRAIL and the mean fluorescence intensities were measured using an intensified camera. Background-subtracted mean florescence intensity for the polystyrene surface was 988.53 ± 47.42 and that for the tube was 684.02 ± 14.72. These results indicate that the polystyrene surface adsorbed more TRAIL than the tube. As indicated by the relative fluorescence intensity, cells resting on the polystyrene surface are exposed to a greater local concentration of TRAIL than those rolling on the capillary flow chamber. From the data, we may conclude that it would take longer than 4 h under static conditions to achieve a 30% kill. Note that HL60 cells exhibit a dose dependent decrease in viability when subjected to immobilized TRAIL (Fig. 4C) although a dose response was not observed with soluble TRAIL (Fig. 2B). Recall that the rolling cells were exposed to the TRAIL surface for only 1 h and resulted in a 30% kill rate while a similar effect was seen at 4 h when resting on a similarly functionalized surface. This observation may be understood by considering the relatively small surface area that is available for the receptors to interact with the surface. Additionally, TRAIL receptors would likely need to diffuse to this small available area and bind to TRAIL before any apoptotic signal is induced, while a rolling cell, over the duration of rolling, presents the entire cell surface to the functionalized device. This eliminates the need for receptor diffusion to the site where TRAIL is present in order to signal apoptosis. Moreover, rolling may be delivering repeated ''on'' signals for apoptosis through periodic ligation and dissociation that may have a cumulative effect and enhance the rate of apoptosis. Thus, a selectin mediated rolling delivery system may ultimately reduce the time to induce the apoptotic signal by fourfold. In another set of experiments, a time-dependent progression of cells from early to late apoptotic stage was observed (data not shown).

Conclusions

In this study, we have demonstrated a novel biomimetic method to capture blood borne metastatic cancer cells from peripheral circulation and induce an apoptotic signal to them without significant effect on healthy cells. With a very short exposure to TRAIL we were able to achieve about 30% kill rate over control. To achieve a similar kill rate under static conditions, the cells must be in contact with the surface for 4 h, thus we have demonstrated a more efficient method of delivering apoptotic signal to metastatic cancer cells. This method may be tailored to specific cancer types by functionalizing the surface with small peptides or other protein molecules that may be more selective at capturing cells. Small molecule replacements would be more stable thereby increasing the shelf life of the device. In addition, the molecules may be customized to capture specific cell types. Cells captured in this manner may then be reprogrammed or neutralized before being released into the circulation. The same technology may be utilized to capture and enrich rare cells from peripheral circulation, which may then be used for clinical research. The proposed device when used as described in this work may significantly reduce metastatic load and potentially improve cancer treatments.

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