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Fluid shear stress sensitizes cancer cells to receptor-mediated apoptosis via trimeric death receptors

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Abstract. Cancer metastasis, the process of cancer cell migration from a primary to distal location, typically leads to a poor patient prognosis. Hematogenous metastasis is initiated by intravasation of circulating tumor cells (CTCs) into the bloodstream, which are then believed to adhere to the luminal surface of the endothelium and extravasate into distal locations. Apoptotic agents such as tumor necrosis factor apoptosis-inducing ligand (TRAIL), whether in soluble ligand form or expressed on the surface of natural killer cells, have shown promise in treating CTCs to reduce the probability of metastasis. The role of hemodynamic shear forces in altering the cancer cell response to apoptotic agents has not been previously investigated. Here, we report that human colon cancer COLO 205 and prostate cancer PC-3 cells exposed to a uniform fluid shear stress in a cone-and-plate viscometer become sensitized to TRAIL-induced apoptosis. Shear-induced sensitization directly correlates with the application of fluid shear stress, and TRAIL-induced apoptosis increases in a fluid shear stress force- and time-dependent manner. In contrast, TRAIL-induced necrosis is not affected by the application fluid shear stress. Interestingly, fluid shear stress does not sensitize cancer cells to apoptosis when treated with doxorubicin, which also induces apoptosis in cancer cells. Caspase inhibition experiments

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reveal that shear stress-induced sensitization to TRAIL occurs via caspase-dependent apoptosis. These results suggest that physiological fluid shear forces can modulate receptor-mediated apoptosis of cancer cells in the presence of apoptotic agents.

Contents

1. Introduction

Approximately 90% of human cancer-related deaths are due to cancer metastasis [1], which consists of a series of discrete steps that allow cancer cell migration from a primary site to a distal location. For hematogenous metastasis to occur, cancer cells must detach from the primary tumor, invade surrounding tissue, and intravasate into the circulation as circulating tumor cells (CTCs) [2]. Once in the circulation, CTCs are believed to adhere to the luminal surface of the microvasculature in a manner similar to the leukocyte adhesion cascade [3]. The process consists of adhesive interactions with the receptor-bearing endothelial cell wall, including selectin-mediated cell tethering and rolling along the endothelium, followed by firm

adhesion or arrest [3, 4]. Once firmly adhered, CTCs may extravasate into a distal site and proliferate to form secondary tumors [5, 6]. Radiation therapy, chemotherapy and surgery are generally successful in the treatment of primary tumors, however the treatment of metastases is challenging due to its systemic nature, and typically signals a poor prognosis [7]. The targeting and treatment of CTCs within the circulation is a potential solution to reduce the probability of metastasis. Several approaches have been developed to isolate patient CTCs from whole blood [8, 9], along with novel strategies to target and treat CTCs with therapeutics under physiological flow [10, 11].

One therapeutic that has displayed potential for the treatment of CTCs is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a type 2 transmembrane protein of the tumor necrosis factor family [12]. TRAIL binds to death receptors (DR4 and DR5) expressed on the surface of a range of cancer cells, which can induce cell apoptosis [13]. TRAIL also binds to three decoy receptors (DcR1, DcR2, DcR3) expressed on the surface of cells, which do not signal apoptosis and can act as competitive inhibitors to apoptosis [14]. Additionally, TRAIL does not exert apoptotic effects on most normal cells [15]. Natural killer (NK) cells, which are believed to play a physiological role in natural protection against tumor formation [16], can express TRAIL on the NK cell membrane. NK cell subpopulations in adult mouse liver constitutively expressed TRAIL in an interferon Υ-dependent manner, and played a role in the suppression of tumor initiation and metastasis [17, 18]. Interferon Υ-dependent TRAIL expression on NK cells also plays a role in interferon Υ-dependent tumor prevention effects of interleukin-12 (IL-12) and α-galactosylceramide (α-GalCer) [19]. In addition to its therapeutic effects as a soluble ligand, TRAIL can play a role in NK cell surveillance of CTCs and tumors [18].

The microenvironments of tumors and CTCs are remarkably different, with fluid shear forces being one factor that is dramatically altered once cancer cells enter the vascular microenvironment. In the tumor microenvironment, cancer cells are exposed to shear stresses created by interstitial flows, which range from 0.1 to 1.0 μm s^{-1} in normal tissues and higher values in the tumor microenvironment [20, 21]. Such flows cause an upregulation of transforming growth factor beta (TGFβ) in fibroblasts, which can lead to myofibroblast differentiation, along with TGFβ-dependent alignment and stiffening of the extracellular matrix [22]. Interstitial flows can also upregulate matrix metalloproteinase expression to enhance glioma cell invasion [23]. Surface shear stress estimates for cancer cells exposed to interstitial flow are difficult to measure, but are estimated to be relatively low in comparison to those experienced in the vasculature. For interstitial flow rates of 1 μm s^{-1}, one study estimated a fluid shear stress range of 0.007–0.015 dyn cm^{-2} [24]. This is in contrast to shear stresses in the circulation, which range from approximately 0.5 to 4.0 dyn cm^{-2} in the venous circulation and 4.0 to 30.0 dyn cm^{-2} in the arterial circulation, with the maximum shear stress experienced at the vessel wall [25]. Increases in fluid shear forces could affect cancer cell survival, as only a small portion of CTCs survive the circulation to generate metastases [26]. Conversely, fluid shear forces can aid CTCs in binding to the vascular endothelium via selectin-mediated tethering and rolling, followed by firm adhesion to the endothelium [27]. While previous studies have investigated the role of fluid shear forces in CTC adhesion to the microvasculature, little is known about the effects of fluid shear forces on the viability and proliferation of CTCs [28].

The role of hemodynamic shear forces in altering receptor-mediated apoptosis of cancer cells has not yet been investigated. In this study, we investigated the role of physiological shear forces in sensitizing cancer cells to TRAIL-mediated apoptosis.

2. Materials and methods

2.1. Cell culture

Colorectal adenocarcinoma cell line COLO 205 (ATCC # CCL-222) and prostate adenocarcinoma cell line PC-3 (ATCC # CRL-1435) were purchased from American Type Culture Collection (Manassas, VA, USA). COLO 205 and PC-3 cells were cultured in RPMI 1640 and F-12K cell culture medium from Invitrogen (Grand Island, NY, USA). Complete media was supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) PenStrep, all purchased from Invitrogen. COLO 205 and PC-3 cells were incubated under humidified conditions at 37 °C and 5% CO_2, and were not allowed to exceed 90% confluence.

2.2. Preparation of cells for fluid shear stress studies

COLO 205 and PC-3 cells were washed in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA, USA) and then treated with Accutase (Sigma Aldrich, St Louis, MO, USA) for 5–10 min at 37 °C before handling. COLO 205 and PC-3 cells were washed with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (DPBS) (Invitrogen) at $300 \times g$ for 5 min at 23 °C. Cells were resuspended in media at a concentration of 0.5×10^6 cells ml^{-1}. Prior to performing fluid shear stress studies, 99% cell viability was confirmed using a trypan blue exclusion stain (Gibco, Grand Island, NY, USA).

For TRAIL studies, cells were treated with 0.1 μg ml^{-1} recombinant human TRAIL (R&D Systems, Minneapolis, MN, USA) prior to the application of fluid shear stress. For caspase inhibition studies, cells were treated with 50 μM of pan-caspase inhibitor Z-VAD-FMK or negative control compound Z-FA-FMK (R&D Systems) for 4 h at 37 °C prior to TRAIL treatment. For doxorubicin studies, COLO 205 cells were treated with doxorubicin hydrochloride (Sigma Aldrich) at a concentration of 20 μM, which has previously been shown to induce COLO 205 cell death, prior to the onset of fluid shear stress [10].

2.3. Cone-and-plate viscometer assay

To study the fluid shear stress response of cancer cells in a controlled, uniform environment, studies were conducted using a cone-and-plate viscometer consisting of a stationary plate underneath a rotating cone maintained at 37 °C by a circulating water bath (Brookfield, Middleboro, MA) as described previously [29]. The design of the cone-and-plate device allows a uniform shear rate to be applied to the cancer cell suspension. The shear rate (G) does not depend on distance from the center of the cone, and is given by

$$G = \frac{\omega}{\tan\theta},$$

where ω is the cone angular velocity (rad s^{-1}) and θ is the cone angle (rad). Under all experimental conditions, a laminar flow field is expected. For a Newtonian fluid under these conditions, the shear stress, τ, is proportional to the shear rate being applied:

$$\tau = \mu G,$$

where μ is the viscosity of the medium. Prior to fluid shear stress experiments, the stationary plate and rotating cone were washed thoroughly with 70% ethanol. TRAIL or doxorubicin-treated cancer cell suspensions were introduced to the plate at a concentration of

0.5×10^6 cells ml^{-1}, and were allowed to equilibrate for 1 min prior to the onset of fluid shear stress. To identify a shear stress threshold required for cancer cell sensitization to TRAIL, cells were exposed to shear stresses ranging from 0.05 to 2.0 dyn cm^{-2} for a duration of 120 min. To determine the shear stress exposure time required for TRAIL sensitization, cells were exposed to a shear stress of 2.0 dyn cm^{-2} for increasing time intervals of 1–120 min. COLO 205 sensitization responses were determined by comparing samples exposed to shear and static conditions using the following equation:

$$\%\,\text{Sensitization} = \frac{(\%\,\text{Cells, shear conditions}) - (\%\,\text{Cells, static conditions})}{(\%\,\text{Cells, static conditions})} \times 100\%.$$

The sensitization equation applies to COLO 205 cells labeled for apoptosis and necrosis, for both TRAIL-treated and untreated samples, at all shear stress magnitudes and exposure times.

After exposure to shear stress, cells were washed thoroughly in PBS and analyzed for cell death using an Annexin-V assay. For doxorubicin studies, cells were washed and incubated overnight prior to apoptosis analysis, as a longer incubation time was required for cells to undergo doxorubicin-induced apoptosis.

2.4. Annexin-V apoptosis assay

A fluorescein isothiocyanate (FITC)-conjugated Annexin-V assay (Trevigen, Gaithersburg, MD, USA) was used to assess cell apoptosis and necrosis. Due to the intrinsic fluorescence of doxorubicin, all doxorubicin treated cells were analyzed using an allophycocyanin (APC)-conjugated Annexin-V assay (BD Pharmingen, San Diego, CA, USA). The manufacturer's instructions were followed to prepare samples for flow cytometric analysis. Viable cells were identified as being negative for both Annexin-V and propidium iodide (PI), early apoptotic cells were positive for Annexin-V only, late apoptotic cells were positive for both Annexin-V and PI, and necrotic cells were positive for PI only.

2.5. Flow cytometry

Cells were incubated with Annexin-V reagents for 15 min at room temperature in the absence of light, and immediately analyzed using an Accuri C6 flow cytometer (Accuri Cytometers Incorporated, Ann Arbor, MI, USA). Flow cytometry plots were generated using Accuri CFlow Plus and FCS Express V3 software (De Novo Software, Thornhill, Canada). The following control samples were used to calibrate the instrument: unlabeled cell samples to evaluate the level of autofluorescence and adjust the instrument accordingly, and cell samples labeled individually with Annexin-V and PI to define the boundaries of each cell population.

2.6. Brightfield and phase contrast microscopy

Cell samples were placed into six well plates and incubated at 37 °C for 60 min to allow cells to adhere to the plate surface. Cells were then imaged by brightfield and phase contrast microscopy using an Olympus IX81 inverted microscope (Olympus America Inc., Center Valley, PA) to observe the presence of viable cells and membrane blebbing, which is characteristic of cells undergoing apoptosis. All images were processed using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

2.7. Death receptor quantification

The average number of death receptors on the surface of cancer cells was determined using flow cytometry calibration with quantum simply cellular (QSC) anti-mouse IgG beads (Bangs Laboratories, Inc., Fisher, IN, USA). QSC beads were incubated for 45 min at 4 °C with a phycoerythrin (PE)-conjugated antibody specific to the antigen on the beads. A mixture of antibody-conjugated beads with a range of antigen binding capacities (ABCs) was run through a flow cytometer. Bead populations corresponding to increasing numbers of ABCs yield increasingly fluorescent peaks in the PE fluorescence channel. Median values of each fluorescence peak were obtained using Accuri CFlow Plus software. Fluorescence data and ABC values reported by the manufacturer were used to generate a calibration curve using QuickCal v2.3 (Bangs Labs, Fisher, IN).

Following the calibration step, surface expression of death receptors DR4 and DR5 on cancer cells was determined using flow cytometry. COLO 205 cells were exposed to static conditions or fluid shear stress (2.0 dyn cm^{-2}) in a cone-and-plate viscometer for 120 min, followed by immediate incubation with either a PE-conjugated isotype or PE-conjugated DR4 and DR5 antibodies (Biolegend, San Diego, CA, USA) for 45 min at 4 °C. Cells were then washed and analyzed for death receptor expression using flow cytometry. Median values of each fluorescence peak were recorded from each sample, and the fluorescence data was converted into the number of receptors using the calibration curve in QuickCal.

2.8. Statistical analysis

Data sets were plotted and analyzed using Prism 5.0b for Mac OS X (GraphPad software, San Diego, CA, USA). A two-tailed paired *t*-test was used for comparisons between two groups with $p < 0.05$ considered significant.

3. Results

3.1. Fluid shear stress increases TRAIL-induced cancer cell apoptosis

We first characterized the effect of fluid shear stress on TRAIL-treated cancer cells in terms of cell viability. COLO 205 cells were treated with TRAIL (0.1 μg ml^{-1}) and then exposed to either static conditions or 2.0 dyn cm^{-2} of fluid shear stress in a cone-and-plate viscometer for 120 min at 37 °C. TRAIL binds to death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) on the cell surface, which signal apoptosis [14]. Both death receptors are expressed on the surface of COLO 205 cells [30]. COLO 205 cells exposed to static conditions (figure 1(A)) or 2.0 dyn cm^{-2} of fluid shear stress (figure 1(B)) for 120 min maintained high cell viability (>94%), with minimal apoptosis observed in the absence of TRAIL (<6%). As expected, COLO 205 cells treated with TRAIL exposed to static conditions for 120 min reduced cell viability by ∼25%, with >22% of the cell population becoming apoptotic (figure 1(C)). However, cells exposed to the same dosage of TRAIL followed by exposure to fluid shear stress (figure 1(D)) induced a greater decrease in cell viability (>53%) and more than doubled the amount of apoptotic cells (>47%), compared to TRAIL-treated samples exposed to static conditions. Experiments performed in triplicate revealed that fluid shear stress alone did not affect cell viability (figure 1(E)) or apoptosis (figure 1(F)), yet induced a significant decrease in cell viability and increase in

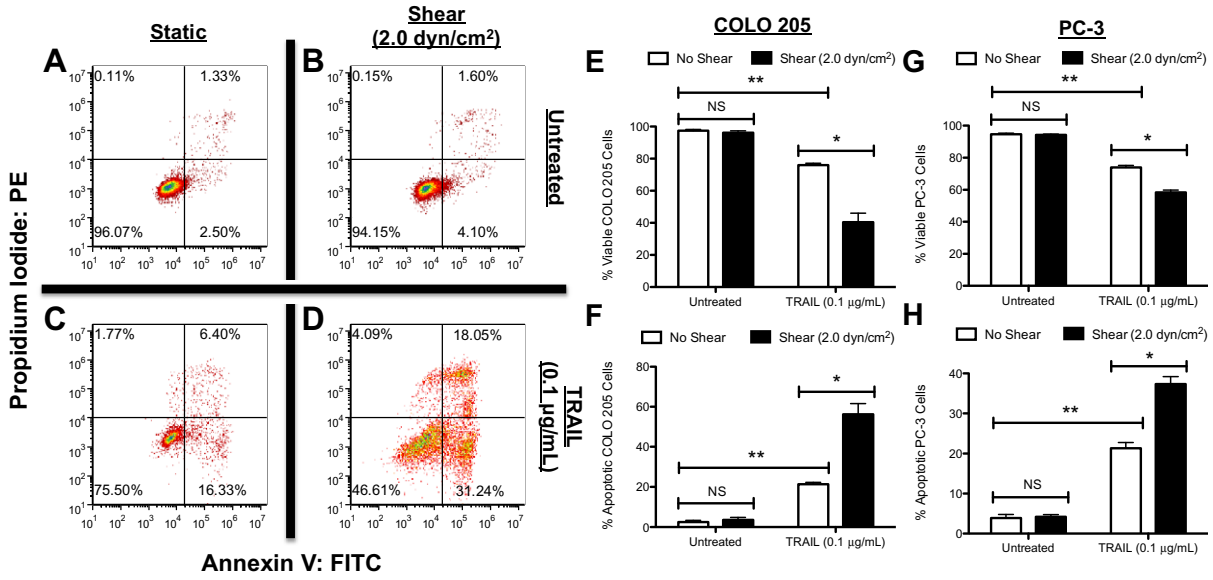

Figure 1. Fluid shear stress sensitizes cancer cells to TRAIL. COLO 205 cancer cells exposed to static conditions (A) and 2.0 dyn cm^{-2} of fluid shear stress (B) for 120 min at 37 °C, respectively. COLO 205 cells treated with 0.1 μg ml^{-1} TRAIL and then exposed to static conditions (C) and 2.0 dyn cm^{-2} of fluid shear stress (D) for 120 min at 37 °C. Per cent viable (E) and apoptotic (F) COLO 205 cells after treatment with 0.1 μg ml^{-1} TRAIL followed by exposure to static conditions and 2.0 dyn cm^{-2} of fluid shear stress ($n = 3$). Per cent viable (G) and apoptotic (H) PC-3 cells after treatment with 0.1 μg ml^{-1} TRAIL followed by exposure to static conditions and 2.0 dyn cm^{-2} of fluid shear stress ($n = 3$). Lower left-hand and right-hand quadrants of each flow cytometry plot represent viable and early apoptotic cells, respectively. Upper left-hand and right-hand quadrants of each flow cytometry plot represent necrotic and late apoptotic cells, respectively. PE: phycoerythrin. FITC: Fluorescein isothiocyanate. Error bars represent 95% confidence intervals. *$P < 0.05$. **$P < 0.01$. NS: non-significant.

apoptosis in the presence of TRAIL. Similar effects on cell viability (figure 1(G)) and apoptosis (figure 1(H)) were also found in experiments performed with prostate adenocarcinoma cell line PC-3. Brightfield microscopy images revealed that COLO 205 cells remained viable and retained their characteristic morphology when exposed to static conditions (figure 2(A)) or 2.0 dyn cm^{-2} of fluid shear stress (figure 2(B)). A greater number of viable cells was observed in TRAIL-treated COLO 205 samples exposed to static conditions (figure 2(C)) compared to TRAIL-treated samples exposed to 2.0 dyn cm^{-2} of fluid shear stress (figure 2(D)), with fewer viable cells and a greater degree of membrane blebbing, characteristic of cell apoptosis. PC-3 cells also remained healthy under static (figure 2(E)) and shear (figure 2(F)) conditions, while a greater number of apoptotic cells was observed in TRAIL-treated samples exposed to shear (figure 2(H)) compared to TRAIL-treated samples exposed to static conditions (figure 2(G)).

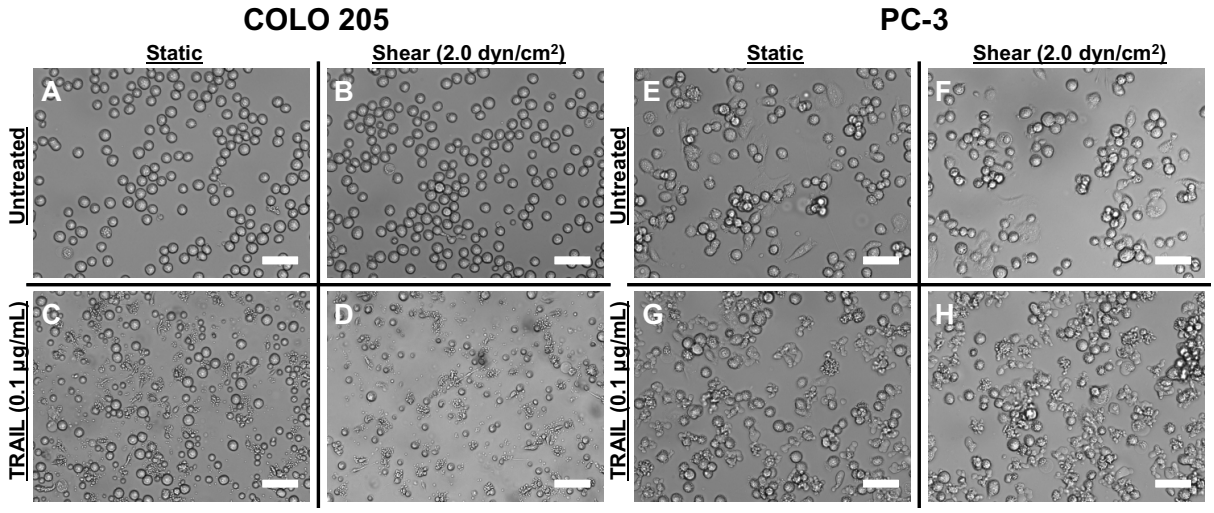

Figure 2. Brightfield microscopy images of untreated COLO 205 cells exposed to static conditions (A) and 2.0 dyn cm^{-2} of fluid shear stress (B) for 120 min at 37 °C. COLO 205 cells treated with 0.1 μg ml^{-1} TRAIL and then exposed to static conditions (C) and 2.0 dyn cm^{-2} of fluid shear stress (D) for 120 min at 37 °C. Untreated PC-3 cells exposed to static conditions (E) and 2.0 dyn cm^{-2} of fluid shear stress (F) for 120 min at 37 °C. PC-3 cells treated with 0.1 μg ml^{-1} TRAIL and then exposed to static conditions (G) and 2.0 dyn cm^{-2} of fluid shear stress (H) for 120 min at 37 °C. Scale bars = 30 μm.

3.2. Fluid shear stress does not alter TRAIL-induced cancer cell necrosis

To assess whether fluid shear stress sensitizes cancer cells to TRAIL-induced necrosis, another form of cell death, cells treated with 0.1 μg ml^{-1} TRAIL followed by shear stress exposure were stained with PI dye and characterized using flow cytometry. Cells positive for PI labeling but negative for Annexin-V were determined to be necrotic, as the cytoplasmic membrane is compromised but lacks the membrane flipping of phosphatidylserine, which is characteristic of apoptosis. Untreated COLO 205 (figure 3(A)) and PC-3 (figure 3(B)) cells exposed to static conditions or fluid shear stress did not show significant differences in necrotic cell death. Treatment with 0.1 μg ml^{-1} TRAIL increased COLO 205 and PC-3 necrotic cell death, compared to untreated samples. However, fluid shear stress did not induce significant differences in TRAIL-mediated COLO 205 and PC-3 necrotic cell death, compared to samples exposed to static conditions. These results indicate that the shear stress sensitization response is TRAIL-mediated *apoptosis*-specific.

3.3. Cancer cell sensitization to TRAIL-induced apoptosis is fluid shear stress dose-dependent

The effect of increasing shear force on cancer cell sensitization to TRAIL-mediated apoptosis was evaluated by exposing TRAIL-treated COLO 205 cells to a range of shear stress from 0.05 to 2.0 dyn cm^{-2}, for an exposure period of 120 min. The shear stress range is representative of shear stress values experienced in the microcirculation [31], and was used to identify a shear stress threshold that induces sensitization to apoptosis. At shear stresses of 0.05 and

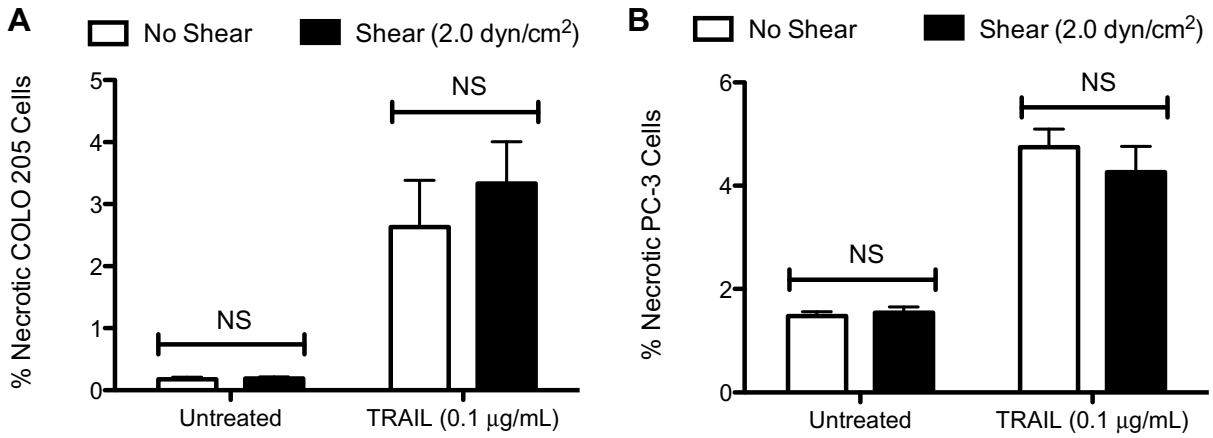

Figure 3. Per cent necrotic COLO 205 cells (A) after treatment with 0.1 μg ml^{-1} TRAIL followed by exposure to static conditions and 2.0 dyn cm^{-2} of fluid shear stress ($n = 3$) for 120 min at 37 °C. Per cent necrotic PC-3 cells (B) after treatment with 0.1 μg ml^{-1} TRAIL followed by exposure to static conditions and 2.0 dyn cm^{-2} of fluid shear stress ($n = 3$) for 120 min at 37 °C. Error bars represent 95% confidence intervals. NS: non-significant.

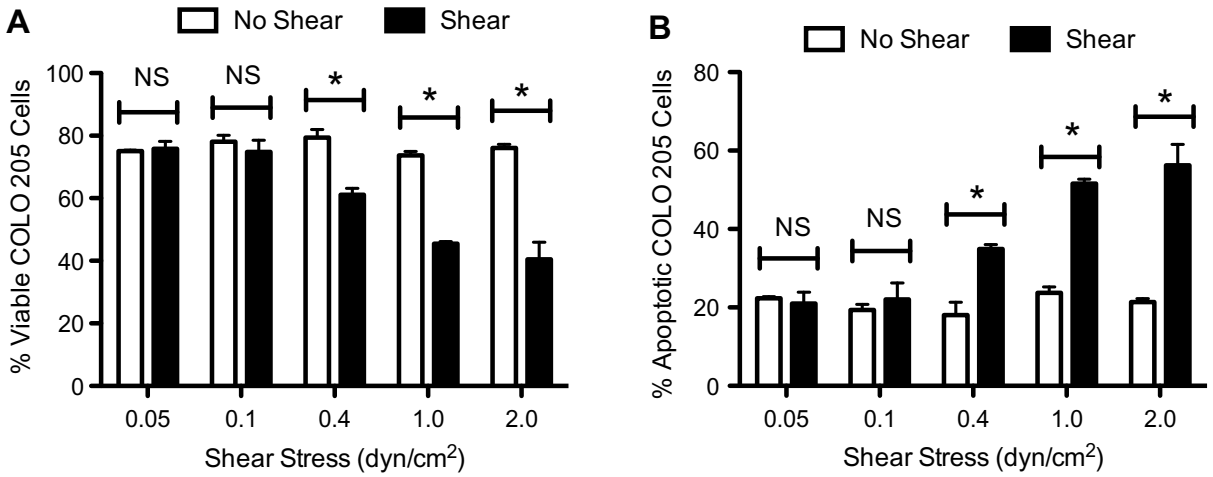

Figure 4. Increasing fluid shear stress sensitizes cancer cells to TRAIL. Per cent viable (A) and apoptotic (B) COLO 205 cells ($n = 3$). Shear stress magnitude was varied in separate experiments from 0.05 to 2.0 dyn cm^{-2} for 120 min at 37 °C. COLO 205 cells were treated with 0.1 μg ml^{-1} TRAIL prior to the onset of fluid shear stress. Error bars represent 95% confidence intervals. $^{*}P < 0.05$ for all measurements.

0.1 dyn cm^{-2}, no significant differences in cell viability or apoptosis were found in TRAIL-treated COLO 205 cells, compared to samples exposed to static conditions. Interestingly, a shear stress of 0.4 dyn cm^{-2} significantly decreased cell viability (figure 4(A)) and increased apoptosis (figure 4(B)), compared to TRAIL-treated cells exposed to static conditions. A shear stress range of 1.0–2.0 dyn cm^{-2} induced a more pronounced decrease in cell viability and increase in cell apoptosis, indicating that the sensitization to apoptosis is fluid shear stress dose-dependent.

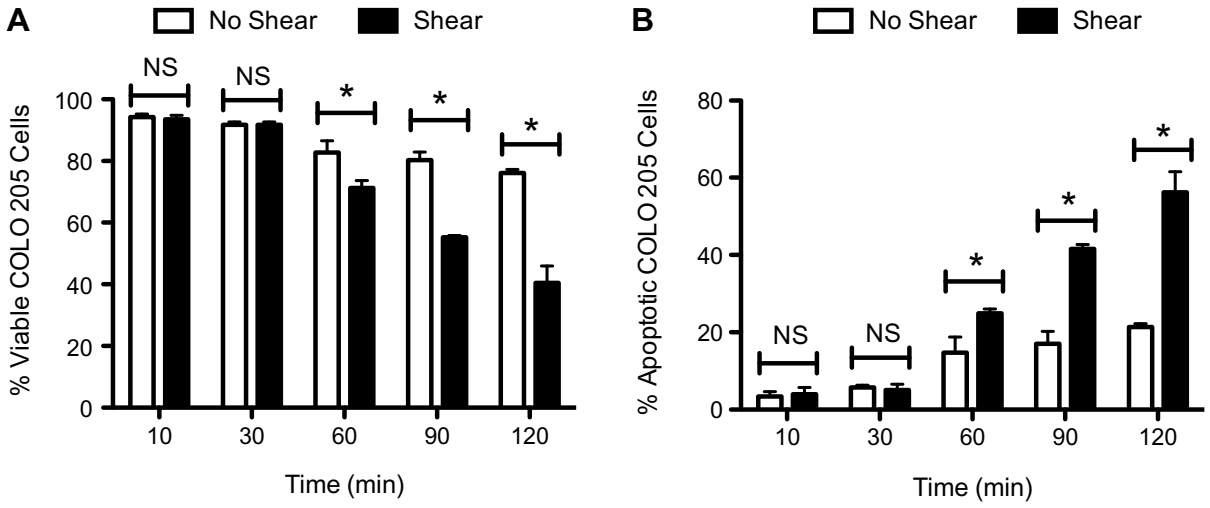

Figure 5. Shear-induced sensitization to TRAIL increases with increasing exposure time to fluid shear stress. Per cent viable (A) and apoptotic (B) COLO 205 cells ($n = 3$). Time dependence of shear-induced sensitization was determined by increasing the fluid shear stress exposure time from 10 to 120 min at a uniform shear stress of 2.0 dyn cm^{-2} at 37 °C. COLO 205 cells were treated with 0.1 μg ml^{-1} TRAIL prior to the onset of fluid shear stress. Error bars represent 95% confidence intervals. $^{*}P < 0.05$ for all measurements.

3.4. Cancer cell sensitization to TRAIL-induced apoptosis is fluid shear stress time-dependent

To assess the kinetics of the sensitization response, TRAIL-treated COLO 205 cells were exposed to a shear stress of 2.0 dyn cm^{-2}, while the fluid shear stress exposure time was increased in parallel experiments from 10 to 120 min. The exposure duration was increased to determine if a threshold time period is required to induce TRAIL sensitization in cancer cells. No significant differences in cell viability (figure 5(A)) and apoptosis (figure 5(B)) were observed in TRAIL-treated COLO 205 cells exposed to fluid shear stress for 10–30 min, compared to samples exposed to static conditions for the same duration. Exposure to fluid shear stress for 60 min significantly decreased COLO 205 cell viability (figure 5(A)) and increased apoptosis (figure 5(B)), compared to TRAIL-treated cells exposed to static conditions. Shear stress exposure times of 90–120 min caused a further decrease in cell viability and increase in COLO 205 apoptosis, providing evidence that the sensitization to TRAIL-induced apoptosis is fluid shear stress time-dependent.

3.5. Cancer cells develop an increasing sensitization to TRAIL-induced apoptosis with increasing shear stress magnitude and shear stress exposure time

Sensitization to TRAIL was quantified by determining the relative difference in COLO 205 cell death for sheared and non-sheared samples, over a range of shear stress magnitudes and exposure times. By varying the magnitude of fluid shear stress, it is apparent that shear stress values of 0.05–0.10 dyn cm^2 induce minimal sensitization of COLO 205 cells to TRAIL (figure 6(A)) as measured by apoptosis, necrosis, and overall cell death. COLO 205 sensitization to TRAIL is readily apparent at a shear stress value of 0.4 dyn cm^{-2}, as cells are sensitized

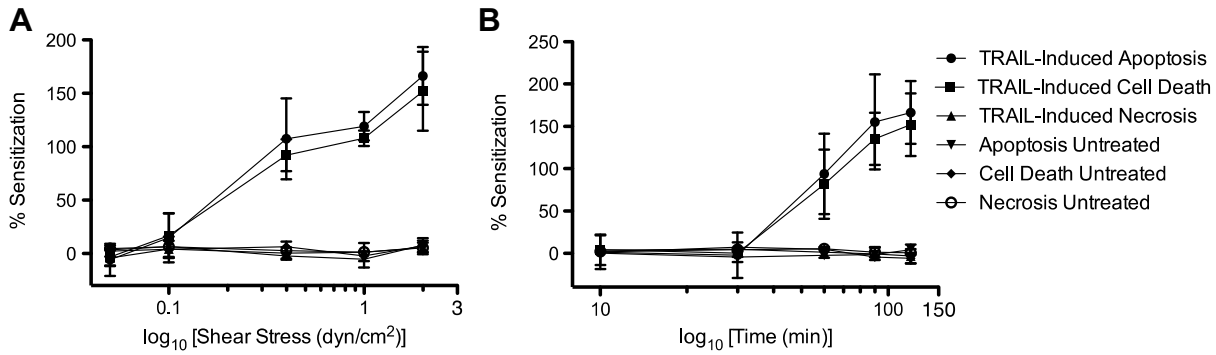

Figure 6. Cancer cells develop sensitization to TRAIL-mediated apoptosis with increasing shear stress magnitude (A) and exposure time (B) ($n = 3$). Resistance is plotted as a function of the $\log_{10}$ of shear stress (dyn cm^{-2}) or $\log_{10}$ of time (min). Error bars represent 95% confidence intervals.

to overall cell death and apoptosis, but not necrosis. Interestingly, sensitization plots also showed that the average per cent sensitization to TRAIL-mediated cell death and apoptosis increased with each increasing shear stress, from 0.4–2.0 dyn cm^{-2} (figure 6(A)). Untreated control samples do not show sensitization to apoptosis, necrosis, and overall cell death across the range of shear stresses.

By varying the exposure time of cells to a fluid shear stress of 2.0 dyn cm^{-2}, a similar trend is observed where short shear stress exposure times of 10 and 30 min do not induce cancer cell sensitization to TRAIL (figure 6(B)). After 60 min of shear stress exposure, COLO 205 cells develop a sensitization to cell death and apoptosis, but not necrosis. From there, sensitization increased linearly with increasing exposure time (figure 6(B)). As expected, untreated control samples do not show sensitization to apoptosis, necrosis, or overall cell death due to fluid shear stress exposure over the time intervals studied.

3.6. Fluid shear stress does not sensitize cancer cells to doxorubicin-induced apoptosis

To assess the effect of fluid shear stress on the cancer cell response to other therapeutics that induce apoptosis, cancer cells were also treated with doxorubicin prior to the onset of fluid shear stress. While TRAIL binds to death receptors on the surface of the cancer cell membrane to signal cell death, doxorubicin induces cell death via inhibition of topo-isomerase II and DNA intercalation [32, 33]. COLO 205 cells analyzed using an APC-conjugated Annexin-V assay showed that untreated COLO 205 cells exposed to static conditions (figure 7(A)) or fluid shear stress (figure 7(B)) do not show measurable differences in apoptotic cell death. Doxorubicin-treated COLO 205 cells experienced an increase in cell apoptosis (22–23%), however minimal differences were found between doxorubicin treated COLO 205 cells exposed to static conditions (figure 7(C)) and fluid shear stress (figure 7(D)). Experiments performed in triplicate revealed no significant differences in untreated cells exposed to static and shear conditions (figure 7(E)), and doxorubicin-treated cells showed no significant differences in apoptosis. Sensitization plots over varying shear stress magnitudes (figure 7(F)) and exposure times (figure 7(G)) show that while a shear-induced sensitization to TRAIL is apparent, COLO 205 cells are not sensitized to doxorubicin treatment upon exposure to fluid shear stress.

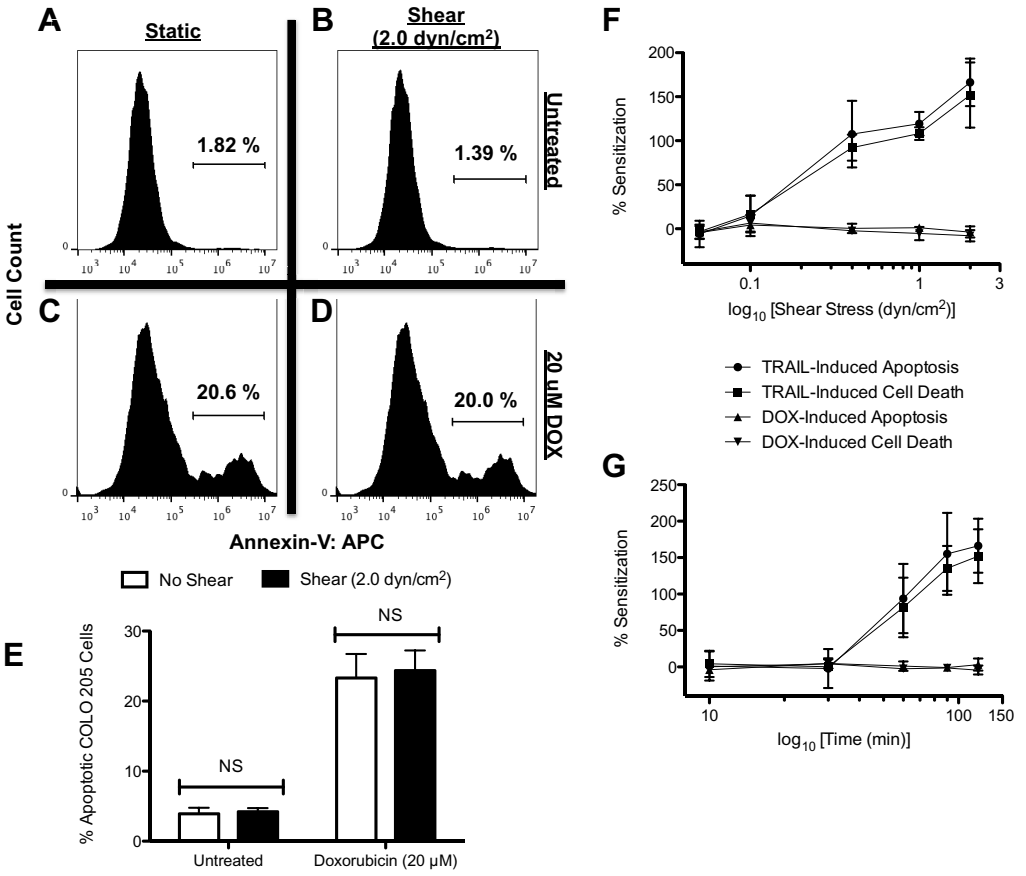

Figure 7. Fluid shear stress does not sensitize cancer cells to doxorubicin. COLO 205 cells exposed to static conditions (A) and 2.0 dyn cm^{-2} of fluid shear stress (B) for 120 min at 37 °C. COLO 205 cells treated with 20 μM doxorubicin and then exposed to static conditions (C) and 2.0 dyn cm^{-2} of fluid shear stress (D) for 120 min at 37 °C. Per cent apoptotic (E) COLO 205 cells after treatment with 20 μM doxorubicin followed by exposure to static conditions and 2.0 dyn cm^{-2} of fluid shear stress ($n = 3$). Comparison of cancer cell shear-induced sensitization to TRAIL and doxorubicin with increasing shear stress magnitude (F) and exposure time (G) ($n = 3$). Resistance is plotted as a function of the $\log_{10}$ of shear stress (dyn cm^{-2}) or $\log_{10}$ of time (min). Error bars represents 95% confidence intervals. Gated region of flow cytometry histograms represents apoptotic COLO 205 cells. Gates determined by labeling viable COLO 205 control samples with Annexin-V APC staining. APC: allophycocyanin. NS: non-significant.

3.7. Cancer cell shear-induced sensitization to TRAIL occurs via caspase-dependent apoptosis

To assess whether shear-induced sensitization to apoptosis is caspase-dependent, COLO 205 cells were incubated with the pan caspase inhibitor Z-VAD-FMK before treatment with TRAIL, followed by exposure to fluid shear stress. The binding of TRAIL to death receptors on the

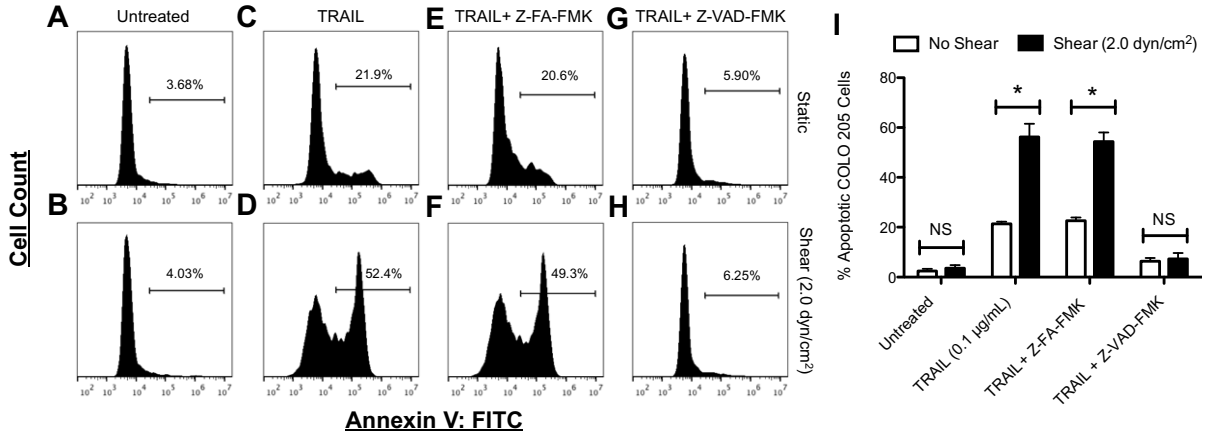

Figure 8. Fluid shear stress sensitization to TRAIL-mediated apoptosis is caspase-dependent. COLO 205 cells (A, B) treated with 0.1 μg ml^{-1} TRAIL (C, D), negative control inhibitor Z-FA-FMK followed by 0.1 μg ml^{-1} TRAIL (E, F), and pan caspase inhibitor Z-VAD-FMK followed by 0.1 μg ml^{-1} TRAIL (G, H), exposed to static conditions and 2.0 dyn cm^{-2} of fluid shear stress for 120 min at 37 °C, respectively. Per cent apoptotic COLO 205 cells ($n = 3$) after exposure to various treatments (I). Gated region of flow cytometry histograms represents apoptotic COLO 205 cells. Gates determined by labeling viable COLO 205 control samples with Annexin-V FITC staining. FITC: fluorescein isothiocyanate. NS: non-significant.

cancer cell surface can activate caspases that initiate the caspase cascade, which triggers cell apoptosis. Z-VAD-FMK is a general caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases, which inhibit apoptosis [34]. FITC-conjugated Annexin-V analysis revealed that untreated COLO 205 cells exposed to static conditions (figure 8(A)) or fluid shear stress undergo minimal cell death, while cells treated with TRAIL showed characteristic sensitization to apoptosis when exposed to fluid shear stress (figure 8(D)), compared to exposure to static conditions (figure 8(C)). While the negative control inhibitor Z-FA-FMK did not affect the sensitization response to TRAIL (figures 8(E) and (F)), treatment with general caspase inhibitor Z-VAD-FMK abolished the sensitization response (figures 8(G) and (H)), as the differences in apoptosis between TRAIL treated samples exposed to shear and static conditions were not significant (figure 8(I)). These results indicate that the shear-induced sensitization to TRAIL is in fact caspase-dependent.

3.8. Fluid shear stress does not alter death receptor expression on the cancer cell surface

To investigate fluid shear stress effects on DR4 and DR5 surface expression, COLO 205 cells were exposed to shear stress (2.0 dyn cm^{-2}) and static conditions at 37 °C for 120 min and immediately labeled with anti-DR4 and anti-DR5 antibodies for flow cytometric analysis. COLO 205 cells exposed to either static conditions or fluid shear stress did not show measurable differences in DR4 (figure 9(A)) or DR5 surface expression (figure 9(B)). QSC bead analysis did not show significant differences in COLO 205 DR4 surface expression, with sheared and non-sheared samples averaging approximately 30 000 receptors per cell (figure 9(C)). COLO

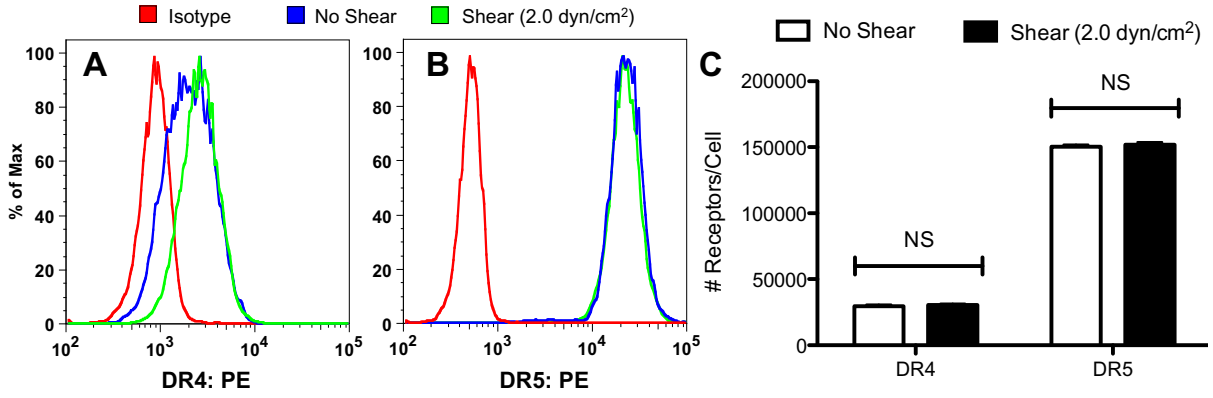

Figure 9. Fluid shear stress does not alter death receptor surface expression. COLO 205 cells exposed to static conditions and 2.0 dyn cm^{-2} of fluid shear stress for 120 min at 37 °C were labeled with anti-DR4 (A) and anti-DR5 (B) antibodies, respectively. (C) QSC receptor quantification of DR4 and DR5 on the surface of COLO 205 cells exposed to static conditions and fluid shear stress ($n = 3$). NS: non-significant.

205 cells also did not show significant differences in DR5 surface expression, with sheared and non-sheared samples averaging approximately 150 000 receptors per cell.

4. Discussion

The aim of this study was to quantify the role of fluid shear stress in altering the cancer cell response to receptor-mediated apoptosis. TRAIL-treated colon and prostate cancer cells were sensitized to receptor-mediated apoptosis under the presence of physiological fluid shear stresses (figures 1 and 2). Previous studies have shown that cancer cells can become chemically sensitized to TRAIL therapy. TRAIL resistant LNCaP cells treated with aspirin have been sensitized to TRAIL treatment via downregulation of NF-κB, a regulator of antiapoptotic proteins [35]. Combined treatment of the demethylating agent 5-Aza-20-deoxycytidine (5-dAzaC) and interferon-Υ (IFN-Υ) sensitize neuroblastoma and medulloblastoma cells to TRAIL-induced apoptosis via upregulation of caspase-8 expression [36]. Second mitochondria-derived activator of caspase (SMAC) synthetic peptides sensitized multiple tumor cell types to TRAIL *in vitro* and enhanced the antitumor effect of TRAIL *in vivo* in a human glioma xenograft model [37]. Our results show that rather than by chemical sensitization, fluid shear forces alone sensitize cancer cells to TRAIL-induced apoptosis.

While fluid shear stress sensitized cancer cells to apoptosis via TRAIL, fluid shear forces did not alter TRAIL-induced cell necrosis (figure 3). TRAIL can induce apoptosis, necrosis, or a combination of both in a variety of cancer cell lines. TRAIL has been shown to induce cell death in prostate adenocarcinoma TRAMP-C2 and Jurkat cell lines via necrosis [38, 39]. In particular, TRAMP-C2 cell death was via necrosis only, as cells lacked apoptotic characteristics such as an annexin V^+/PI^- population, SAPK/JNK phosphorylation, caspase activation, or cytochrome *c* release [38]. Recently, acidic extracellular pH has been shown to alter the form of TRAIL-induced cell death, from apoptosis to receptor interacting protein kinase 1 (RIPK1)-dependent regulated necrosis in colon adenocarcinoma HT29 and hepatocarcinoma HepG2 cell lines

[40, 41]. Shear-induced sensitization to TRAIL did not show a shift from TRAIL-induced apoptosis to TRAIL-induced necrosis, indicating that the sensitization response is apoptosis-specific.

Cancer cells developed a shear-induced sensitization to TRAIL-induced apoptosis in a fluid shear stress force- and time-dependent manner, directly implicating fluid shear stress in this response (figures 4 and 6). While low shear forces representative of those generated by interstitial flows did not sensitize cancer cells to TRAIL, a minimum shear stress of 0.4 dyn cm^{-2} induced a significant increase in TRAIL-induced apoptosis. In the tumor microenvironment, cancer cells are exposed to slow interstitial flows in and around the tumor tissue [42]. The mechanisms behind how cancer cells sense interstitial flow are not well understood, however shear stress values have been estimated in three-dimensional *in vitro* matrices [24]. For flow rates of 1 $\mu m\,s^{-1}$, cell surface shear stress estimates are extremely low, ranging from 0.007–0.015 dyn cm^{-2} [24]. Cancer cells are exposed to greater fluid shear forces upon entering the circulation, and such conditions may play a role in sensitization to TRAIL-induced apoptosis. It is interesting to note that the cone-and-plate viscometer shear experiments were designed so that fluid shear forces alone would not induce significant cancer cell death, compared to cancer cells exposed to static conditions. Thus, we were able to isolate fluid shear stress effects on receptor-mediated apoptosis, implicating shear-induced sensitization to TRAIL as a synergistic response.

While fluid shear stress sensitized cancer cells to TRAIL-induced apoptosis, cancer cells did not show an increase in doxorubicin-induced apoptosis under the presence of fluid shear forces (figure 7). Much like TRAIL, chemical sensitization to doxorubicin has been investigated previously. Gliotoxin, MG132, and sulfasalazine sensitized typically resistant pancreatic carcinoma Capan-1 and A818-4 cell lines to doxorubicin-induced apoptosis via inhibition of NF-κB [43]. Selenium treatment combined with doxorubicin was successful in enhancing apoptosis in MCF-7 breast cancer cells, a doxorubicin-resistant cell line, via depression of Akt phosphorylation [44]. Small molecule inhibitors of the Hdm2:p53 complex, allowing for activation of tumor suppressor p53, exerted synergistic effects with doxorubicin in an A375 melanoma cell line xenograft model to decrease tumor growth [45]. Due to the fact that shear stress-induced sensitization to apoptosis was not observed with doxorubicin treatment, it is possible that the fluid shear stress effects originate at the cell surface receptor level, where TRAIL ligand binds to death receptors DR4 and DR5 while exposed to fluid shear stress. This is in direct contrast to doxorubicin, which interacts with DNA within the cell to exert its apoptotic effects.

Treatment with Z-VAD-FMK revealed that shear-induced sensitization to TRAIL-induced apoptosis is caspase-dependent (figure 8). Caspase activation is a critical step in the apoptotic pathway, induced by TRAIL binding to death receptors [46]. In contrast to extrinsic apoptosis pathways such as TRAIL-mediated apoptosis, intrinsic pathways are initiated by DNA and cellular damage, along with the permeabilization of mitochondria [47]. During this process, mitochondrial factors including cytochrome *c*, AIF (apoptosis-inducing factor), and SMAC are released, with AIF-induced apoptosis occurring via a caspase-independent process [48]. DNA-damaging agents have previously been shown to sensitize hepatic carcinoma cell lines to TRAIL, due to ATM kinase activation [49]. ATM kinase activity in turn leads to a downregulation of antiapoptotic protein cFLIP, and subsequent sensitization to TRAIL. Since our sensitization process is caspase-dependent, it is likely that the shear-induced sensitization is not due to DNA-damaging events, providing further support that the sensitization phenomena

may occur at the cell surface. Inhibition of WEE1, a cell cycle checkpoint regulator, has been shown to sensitize a variety of basal breast cancer cell lines to TRAIL-induced apoptosis due to increased surface expression of death receptors and increased caspase activation [50]. Our results show that COLO 205 surface expression of death receptors DR4 and DR5 is not altered after exposure to fluid shear stress (figure 9), and thus sensitization to TRAIL-induced apoptosis is not likely due to shear-induced changes in receptor expression. It is likely that a combination of fluid shear stress effects along with TRAIL stimulation, rather than fluid shear stress alone, cause changes in death receptor trimerization and signaling. Death receptors, upon binding to TRAIL, are known to trimerize and recruit adaptor proteins to form a signaling complex required for TRAIL-induced apoptosis [51]. It is possible that mechanical shear forces could enhance death receptor trimerization in the presence of TRAIL, and assist in the formation of signaling complexes for TRAIL-induced apoptosis. The effects of fluid shear stress on death receptor trimerization upon binding to TRAIL could lead to further insight into the mechanistic basis of shear stress-induced TRAIL sensitization.

5. Conclusion

Results from this study indicate that hemodynamic shear forces have a significant effect on receptor-mediated apoptosis of cancer cells in the presence of TRAIL. Fluid shear stress was found to sensitize both colon and prostate cancer cell lines to TRAIL-mediated apoptosis. Cancer cells were not sensitized to TRAIL-mediated necrosis upon exposure to fluid shear stress. TRAIL sensitization was shown to be shear stress dose-dependent, as sensitization was found to increase with increasing fluid shear stress. TRAIL sensitization was also fluid shear stress time-dependent, as sensitization to apoptosis was enhanced with increasing fluid shear stress exposure time. The response was TRAIL-specific, as shear stress did not sensitize cancer cells to doxorubicin treatment over varying shear stress magnitudes and exposure times. Caspase inhibition assays revealed the sensitization response to be caspase-dependent. These results shed new light on the cancer cell response to soluble apoptotic agents within the circulation. The effects of fluid shear stress on mechanosensing death receptors on the cancer cell surface, along with their signaling pathways, can reveal new strategies for treating circulating cancer cells and reducing the likelihood of metastasis.

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