Unnatural killer cells to prevent bloodborne metastasis: inspiration from biology and engineering

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Metastasis contributes to over 90% of cancer-related deaths. Many types of cancer metastasize via the bloodstream, where circulating tumor cells (CTCs) originating from the primary tumor can undergo selectin-mediated adhesion with the blood vessel wall and subsequently transmigrate to anatomically distant organs. In an effort to neutralize CTCs with the potential to form metastases, a new therapeutic approach has been developed in which circulating leukocytes are functionalized to target and kill cancer cells in the bloodstream. This approach mimics the cytotoxic activity of natural killer cells and the chemical engineering concept of a fluidized bed reactor, which increases the surface area for surface-catalyzed reactions. The resulting 'unnatural killer cells', proven effective *in vitro* with human blood and also in the living mouse, holds promise in neutralizing CTCs to interrupt the metastasis process.

Metastasis remains the cause of over 90% of cancer-related deaths [1]. A complex series of steps are necessary for metastasis to occur: cancer cells from the original tumor site invade surrounding tissues, where they can then squeeze through the blood vessel wall and enter the bloodstream as circulating tumor cells (CTCs). CTCs must then survive the harsh shear forces of the circulation [2,3] to adhere within the blood vessels of distant tissues, where they can migrate back into tissue, adapt and proliferate in a foreign microenvironment and form a secondary tumor [4]. When constrained to the primary tumor site, surgery, radiation and/or chemotherapy have proven to be effective cancer treatments. However, the disease becomes extremely difficult to detect and treat once CTCs progress to form micrometastases in distant organs, making the prevention of metastasis a crucial step in the effective treatment of many cancer types.

In recent years, much progress has been made in understanding the physical translocation of CTCs via the bloodstream as a key step of metastasis. Similar in mechanism to how white blood cells migrate from blood into tissue during inflammation and infection [5], CTCs have been shown to interact with the blood vessel wall via receptor-mediated adhesion [6]. CTCs from primary tumors originating from multiple organs express sialylated carbohydrate ligands, similar to the adhesion molecules of leukocytes, which mediate interactions with E-selectin (ES) on the endothelium [6]. Selectins possess rapid, force-dependent binding kinetics, which can trigger the rolling adhesion of CTCs along the blood vessel wall under flow [7]. CTCs can subsequently transition from rolling to firm adhesion, allowing for transendothelial migration into tissues and eventual formation of metastases [8]. In an attempt to target and kill CTCs to prevent metastases, our laboratory has previously sought to exploit these CTC interactions with ES by developing implantable devices coated with ES and various chemotherapeutics [9,10] and cell death-inducing ligands [11].

The translatability of these implantable devices has been challenged by the fact that white blood cells, which can outnumber CTCs by a million to one,

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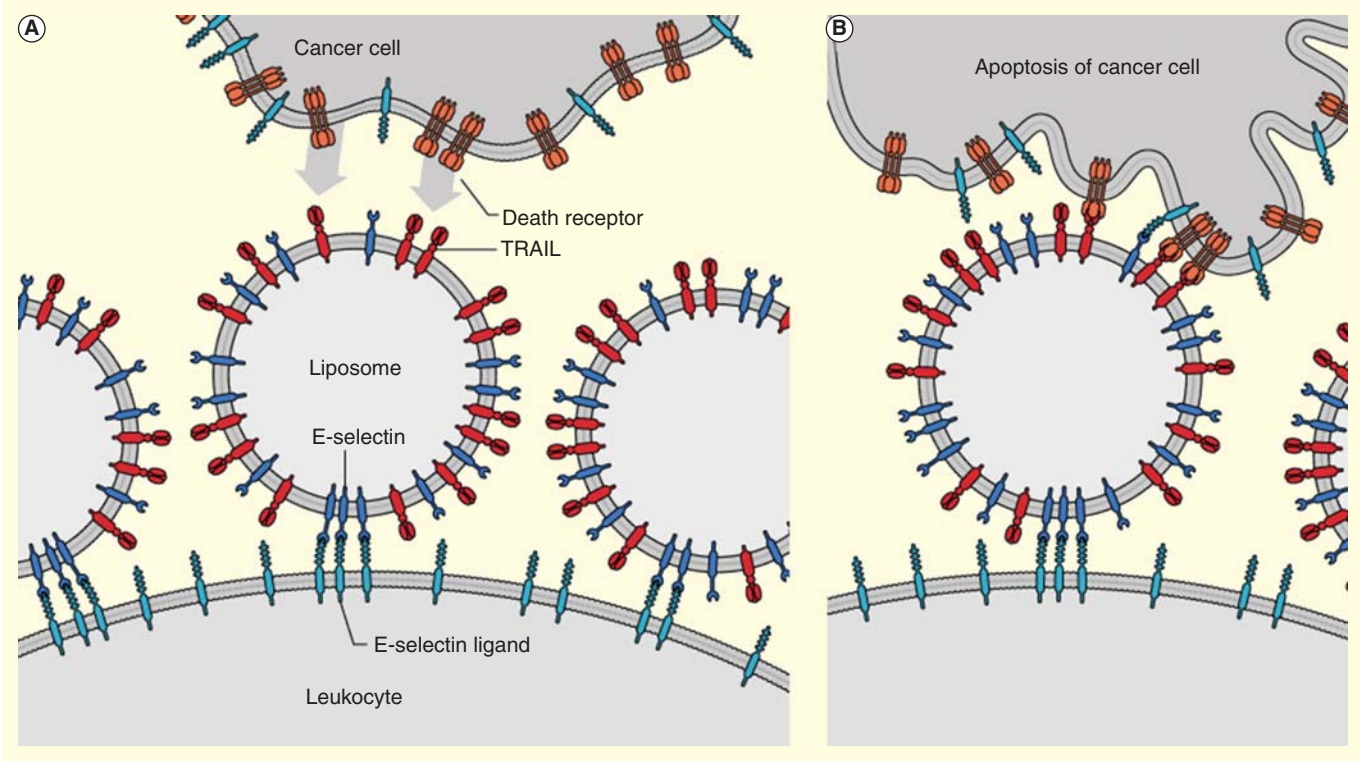

Figure 1. Schematic of the two-step mechanism utilizing UnKs to target and induce circulating tumor cell apoptosis in the bloodstream. (A) Upon injection into the bloodstream, leukocytes possessing E-selectin ligands adhesively interact with ES/TRAIL liposomes to form UnKs. **(B)** UnKs then contact CTC and activate death receptors via TRAIL, to induce CTC apoptosis.
ES: E-selectin; TRAIL: TNF-related apoptosis-inducing ligand; UnKs: Unnatural killer cells.
Adapted from [13].

also possess molecules that adhere to ES. Thus, the therapeutic employed on the device surface would largely interact with white blood cells, limiting CTC interaction with therapeutics. In fact, CTCs are essentially surrounded by white blood cells in the circulation, as deformable red blood cells tend to flow toward the center of the blood vessel while white blood cells and CTCs are pushed toward the vessel wall during a process known as margination [12]. With this in mind, we developed a reverse approach, utilizing the surface of the body's white blood cells to create 'unnatural killer cells' (UnKs) to target and deliver a cell death signal to CTCs in blood [13].

Since both white blood cells and CTCs adhere to ES in blood, we developed nanoscale liposomes conjugated with ES, to mediate interactions between both cell types, and TNF-related apoptosis-inducing ligand (TRAIL), a therapeutic that induces cell death in numerous cancer cell types while exerting minimal toxic effects on normal cells (Figure 1) [14]. *In vitro*, liposomes functionalized with ES and TRAIL (ES/TRAIL) were generally effective at targeting cancer cells and inducing apoptosis under both static conditions and flow conditions mimicking the circulatory system. Additionally, ES/TRAIL liposomes were found to adhere to a range of white blood cells isolated from human blood under flow conditions *in vitro*, demonstrating that UnKs could target CTCs in a complex fluid such as blood. The results were surprising and unexpected, however, as human blood *enhanced* the therapeutic effect of ES/TRAIL, with minimal live cancer cells remaining in human blood after exposure to flow conditions for as little as 2 h *in vitro*.

The presence of whole blood enhanced the therapeutic effect of ES/TRAIL in two ways: the presence of red blood cells and the formation of UnKs after treatment with ES/TRAIL liposomes under flow. By simply increasing the number of red blood cells from low levels to normal hematocrit levels found in blood, the therapeutic effect of ES/TRAIL exerted on cancer cells significantly increased. Perhaps most interesting, however, was utilizing UnKs alone to target cancer cells under blood flow conditions. By adhering ES/TRAIL liposomes to surface of white blood cells and removing unbound ES/TRAIL liposomes from blood, the maximal therapeutic effect was still achieved, demonstrating UnKs as the essential component for killing circulating cancer cells.

The approach is inspired by both the cytotoxic activity of natural killer cells, which can be induced to present TRAIL and participate in immunosurveillance, and the chemical engineering concept of fluidized bed reactor, which increases surface area for surface-catalyzed interactions. A fluidized bed reactor consists of dense particles suspended in a fluid to maximize surface area on which to carry out a surface-catalyzed reaction [15]. In a sense, the introduction of ES/TRAIL liposomes into the blood converts the entire peripheral circulation into one contiguous fluidized bed. The surface reaction in this case

is TRAIL binding reversibly to TRAIL death receptors on cancer cells and is facilitated by ES binding to cancer cell selectin ligands. Interestingly, the specific surface (surface area per volume) for leukocytes in blood, at a concentration of 4 million cells/mL, is approximately 800 m^2/m^3, which exceeds the range of industrial Raschig rings used to maximize surface area in packed bed reactors (50–400 m^2/m^3, depending on ring size). Near the blood vessel wall, where margination elevates the local concentration of white blood cells and CTCs above systemic values by several fold, the specific surface of liposome-coated white blood cells will far exceed such engineered systems.

UnKs have numerous benefits for targeting CTCs in the circulation *in vivo*. From a biophysical perspective, the collisions between CTCs and white blood cells can act to flatten the matrix of biological molecules that make up the CTC glycocalyx [16], allowing for exposure of TRAIL death receptors that would not be possible to target using nanoparticles alone. Additionally, while TRAIL alone is not effective means to target CTCs due to its short half-life *in vivo* [17], the formation of UnKs in the circulation acts to increase TRAIL circulation time, effectively avoiding renal clearance mechanisms to enhance the therapeutic effects on CTCs *in vivo*.

Initial *in vivo* experiments to target CTCs in the circulation have proven to be similarly promising. ES/TRAIL liposomes were injected into the peripheral circulation of mice for 30 min, allowing for UnKs to form in blood. As a straightforward and widely used model of lung metastasis *in vivo* [18], fluorescently labeled cancer cells were injected via tail vein and allowed to circulate for 2 h, after which the mice were sacrificed and cancer cells were recovered from blood via cardiac puncture. Interestingly, mice with UnKs showed >98% less cancer cells in blood than control mice that only displayed ES on the white blood cell surface. Injection with the soluble TRAIL alone was ineffective, due to its short half-life in the circulation. In this model of lung metastasis, it is known that cancer cells also lodge within the blood vessels of the lung. Thus, we utilized multiphoton microscopy to assess the number and viability of cancer cells lodged within the lung. Interestingly, fewer cancer cells were found in mice with UnKs, indicating that most cancer cells underwent apoptosis and dissociated by the time of imaging. Of the cancer cells remaining in UnK mice, the majority of the cells stained positive for the apoptosis marker Annexin-V, while a minimal number of cells in control mice were apoptotic. Thus, the formation of UnKs in mice suggests that cancer cells within the circulation can be targeted and killed, before they are able to form new micrometastases.

The use of UnKs to target and kill bloodborne cancer cells has the potential to be translated into the clinic, as Amgen/Genentech's recombinant human TRAIL/Apo2L (also known as PRO1762) has undergone Phase I, Ia, II and III clinical trials over the past decade, with minimal adverse effects reported [19]. Given that this new therapeutic approach is focused on the vascular microenvironment, we also validated that cells that comprise the circulation such as leukocytes and endothelial cells remain unharmed by this approach [13]. In fact, most normal cells in the body are protected from TRAIL-induced cell death through intracellular proteins such as the class of inhibitors of apoptosis proteins [14]. For CTCs that exhibit reduced sensitivity to the effects of TRAIL, the approach may also be combined in regimens with other low cytotoxic agents, such as aspirin or the turmeric spice compound curcumin [11,20], to synergistically kill CTCs.

If introduced at the appropriate time points, UnKs may serve as a means to target and kill CTCs before they are able to form new metastases. Such an approach could constrain cancerous cells within the primary tumor, which is typically treatable via surgery, radiation and/or chemotherapy. Future work focused on the prevention of metastases in longer term animal models will be needed to address the translatability of UnK therapy into future clinical trials. Furthermore, combinatorial approaches utilizing UnK cells in combination with compounds that demonstrate minimal side effects *in vivo* can be used to expand this approach to cancers which show differential sensitivity to TRAIL. We believe that the current approach, however, represents an important initial step in utilizing white blood cells within the bloodstream to eliminate CTCs *in vivo*.

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