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## Tissue factor-bearing microparticles and cancer-associated thrombosis

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**M**icroparticles were first described in normal blood as *platelet dust*.<sup>1</sup> Sims carefully studied the generation of platelet microparticles during platelet activation by complement.<sup>2</sup> These platelet microparticles bind to Factor Va and support prothrombinase activity<sup>3</sup> as well as bind to Factor VIII.<sup>4</sup> Since these reports, hundreds of studies have monitored microparticles, primarily using flow cytometry and light scattering, and correlated microparticle antigen expression within diverse groups of diseases. However, microparticles have been subjected to limited chemical analysis, including their lipid and protein content.

Microparticles derived from platelets, endothelial cells, and leukocytes have been shown to exhibit procoagulant properties. Although the specific quantitation, sizing and identification of these microvesicles in a variety of disease states may not be accurate, numerous groups have reported their correlation as biomarkers of ongoing coagulation. In some cases, tissue factor associated with microparticles have been implicated in the procoagulant activity.<sup>5-8</sup> Platelet-derived microparticles have also been thought to provide a procoagulant surface for thrombin generation because of the richness of phosphatidylserine<sup>2, 9, 10</sup> as well as a surface for the assembly of protein complexes associated with blood coagulation.<sup>3, 4</sup> Endothelial cell-derived microparticles have been proposed to be associated with hypercoagulable states, including thrombotic events associated with the lupus anticoagulant,<sup>11</sup> thrombotic thrombocytopenic purpura,<sup>12</sup> sickle cell disease,<sup>13</sup> and preeclampsia.<sup>14</sup>

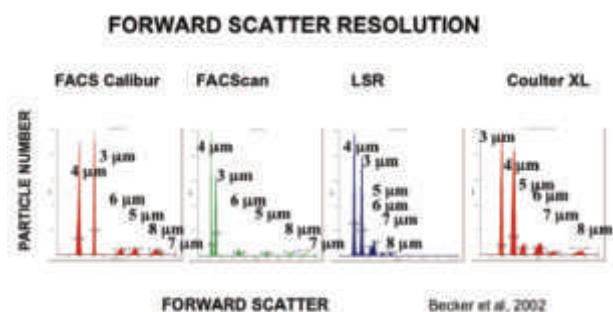
The association between tumor shedding of microparticles and the hypercoagulable state of cancer was first made by Dvorak almost 25 years ago.<sup>15</sup> A guinea pig hepatocarcinoma cell line and a mouse breast carcinoma cell line released procoagulant activity in tissue culture and *in vivo*. In each case, procoagulant activity was associated with the shedding of sedimentable mem-

brane-derived microvesicles. No procoagulant activity remained in the supernatant following ultracentrifugation. The pellet contained membrane vesicles lacking any visible content by electron microscopy. A wide variety of tumor cells of human, mouse and guinea pig origin released procoagulant activity within small vesicles shed from the cell surface.<sup>16</sup> This procoagulant activity behaved in a fashion consistent with its identity with tissue factor. These reports of microparticle procoagulant activity predated the purification of tissue factor<sup>17</sup> and the development of immunochemical reagents for its identification and characterization on cancer cells.

Since this initial report, many studies have described shedding of procoagulant microvesicular structures from tumor cells. Tissue factor was shown to be shed from human glioblastoma cells and induce coagulation, platelet aggregation and thrombus formation.<sup>18</sup> Membrane vesicles were found in certain types of leukemia, including acute promyelocytic leukemia, acute myelogenous leukemia, and acute monocytic leukemia.<sup>19</sup> These vesicles shared antigens with leukemic cells. Some of these vesicles expressed procoagulant activity, but it was not possible to correlate procoagulant activity of these vesicles with the clinical coagulation disorders in these patients.

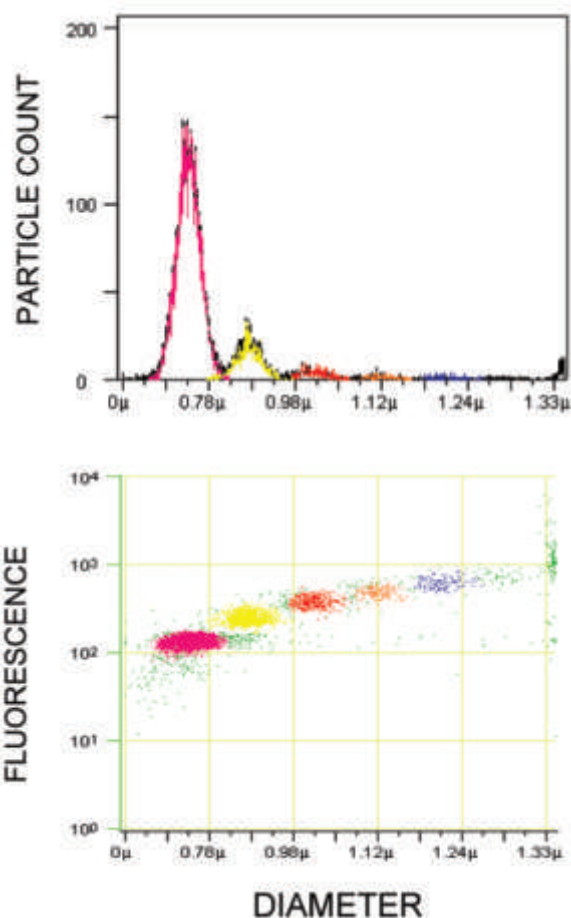
Although tissue factor on shed tumor microparticles had been implicated in the induction of a prothrombotic state in patients, microparticles have also been demonstrated to support the assembly of blood coagulation protein complexes in the absence of tissue factor. For example, membrane vesicles, shed from guinea pig and human tumor cells in culture, supported the assembly of functional prothrombinase activity.<sup>20</sup> Thus, some microparticles may not initiate blood coagulation but rather support the generation of thrombin by providing a surface for the generation of fibrin.

The detection, quantitation and sizing of microparticles has been very problematic.



**Figure 1. Histogram of particles analyzed by forward light scattering using commercial flow cytometers. Particles are beads accurately defined in size: 3, 4, 5, 6, 7, 8 micrometers in diameter. Particle number, y axis; forward light scatter, x axis. Forward scatter is not related directly to particle size. Modified from Becker et al, 2002.<sup>21</sup>**

Despite significant interest in blood microparticles as biomarkers for specific diseases, most investigators have used flow cytometry and light scattering as the principle methodology. Although flow cytometry is facile and offers great opportunity for monitoring antigens on cell surfaces where cells, such as white blood cells, have a diameter of 10  $\mu\text{m}$ , even platelets, at 1–2  $\mu\text{m}$ , can be difficult to size. Microparticles, generally defined as microvesicles less than 1  $\mu\text{m}$  in diameter, appear with the electronic noise and cellular debris in the first decile of the light scatter report. Flow cytometry is widely available to investigators who are not aware of its limits, and thus great controversy exists in the biomedical literature of microparticles because of the application of this technology. These studies have ignored that one cannot measure the relative size of two particles of different type from small angle light scatter signals because the presence of strongly absorbing material in these particles decreases the amplitude of forward scatter signals. Similarly, textured surfaces and internal structures also modulate the amplitude of forward scatter signals. The relationship of forward scatter intensity and the size of polystyrene beads of various diameters is not monotonic i.e. the signals from bigger beads are not necessarily larger than the signals from smaller beads.<sup>21</sup> The critical point, well known to those who study the physics of flow cytometry, is that particle size can not be measured using light scattering when the incident light has a wavelength that is in the same order of magnitude as the particle diameter. Since laser light of 488 nm is usually employed and microparticles vary in size below 1000 nm in diameter, the sizing of microparticles is not accurate and even the identification of microparticles is not accurate. To make this point, Becker<sup>21</sup> took beads of 3, 4, 5, 6, 7, and 8  $\mu\text{m}$  in diameter, accurate to  $\sim 5\%$  as defined by electron microscopy, and analyzed these beads for size in four



**Figure 2. Calibration beads analyzed by impedance-based flow cytometry. Beads (780 nm) were analyzed. Upper panel: x axis, particle diameter; y axis, particle count. Lower panel: x axis, particle diameter; y axis, particle number.**

standard laboratory flow cytometers (Figure 1). These instruments could not size these beads to better than  $\sim 2000$  nm and indeed could not even determine the size order of the six beads. Thus, light scattering is not an adequate approach to study microparticles. The use of light scattering to measure particle size has greatly confused the literature in this field and is likely the basis for the absence of consensus in the use of microparticle measurements as biomarkers of specific disease states.

By *in vivo* imaging of thrombus formation in real time in the living mouse,<sup>22</sup> we have demonstrated that microparticles expressing tissue factor and PSGL-1 accumulate in the developing thrombus.<sup>23</sup> P-selectin expressed on activated platelets in the platelet thrombus capture microparticles via PSGL-1, thus leading to the accumulation of tissue factor in the thrombus. More recently using bone marrow transplant chimeras of low tissue factor mice and wild type mice, we have

shown that tissue factor on microparticles contribute significantly to fibrin formation in the thrombus.<sup>24</sup> These leukocyte microparticles circulate constitutively in the blood although their appearance may increase during the inflammatory response or perhaps apoptosis. P-selectin interaction with leukocytes may lead to increases in microparticle concentration.<sup>8</sup> Whether tissue factor associated with leukocyte microparticles or tissue factor associated with tumor microparticles are important in the hypercoagulable state associated with malignant diseases remains unknown.

Thromboembolic disease is well recognized as a major complication of cancer, and is associated with pulmonary embolism, deep venous thrombosis, superficial thrombophlebitis and clotting of central venous catheters. We have evaluated the hypothesis that elevated numbers of tissue factor-bearing microparticles might be one of the causes of cancer-associated thrombosis. Since the light scattering methodology used in commercial flow cytometers can not determine particle size when the diameter of the particle is the same order of magnitude as the wavelength of the incident light employed, we have applied novel instrumentation to determine the size, size distribution, and concentration of tissue factor-bearing microparticles. An NPE Systems Quanta flow cytometer, using impedance to measure particle size, was modified extensively for this microparticle application. Modifications included introduction of a small flow cell, alteration of the electronics for improvements in signal sensitivity

and decreased noise, optimization of fluidics and valve control, and ultrafiltration of buffers to eliminate particulates. Application of 780 nm diameter fluorescent calibration beads yielded a major population from 740 to 820 nm (Figure 2). Application of 520 nm diameter fluorescent beads yielded a major population from 500 to 540 nm. Using a high affinity antibody to tissue factor, tissue factor-bearing microparticles were measured in platelet-poor plasma derived from normal patients and patients with advanced cancer by impedance-based flow cytometry. In preliminary studies, we have established that tissue factor-bearing microparticles are found in about one-third of patients with advanced cancer, including patients with pancreatic carcinoma, ovarian carcinoma, colon cancer, breast cancer. Tissue factor-bearing microparticles were below the level of detection in our system in normal subjects. In positive samples, tissue factor microparticle concentrations varied between 11,000 particles per microliter to 1,600,000 particles per microliter. The mean diameter of tissue factor microparticle size varied from 332 nm to 501 nm. These results indicate that tissue factor microparticles are present in a spectrum of cancer patients that are known to have a high incidence of thromboembolic complications. Continued studies will establish whether high concentrations of tissue factor microparticles predict thromboembolic complications and whether tissue factor microparticles are central to the pathogenesis of cancer-associated thrombosis.

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