Circulating microvesicles in B cell chronic lymphocytic leukemia can stimulate marrow stromal cells: implications for disease progression

Running Title: CLL microvesicles can activate stroma

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ABSTRACT

Microvesicles (MV) released by malignant cancer cells constitute an important part of the tumor-microenvironment as they can transfer various messages to target cells and may be critical to disease progression. Here, we demonstrate that MV circulating in plasma of B-CLL patients exhibit a phenotypic shift from predominantly platelet-derived in early stage to leukemic B-cell derived at advanced stage. Furthermore, the total MV level in CLL was significantly higher compared to normal subjects. In an effort to understand the functional implication of the elevated MV level in CLL, we examined whether MV can interact and modulate CLL bone marrow stromal cells (BMSC) known to provide a “homing and nurturing” environment for CLL B-cells. We found that CLL-MV can activate the AKT/mTOR/p70S6K/HIF-1α axis in CLL-BMSC with production of VEGF, a survival factor for CLL B-cells. Moreover, MV-mediated AKT activation led to modulation of the β-catenin pathway and increased expression of cyclin D1 and c-myc in BMSC. We found MV delivered phospho-receptor tyrosine kinase Axl directly to the BMSC in association with AKT activation. This study demonstrates the existence of separate MV phenotypes during leukemic disease progression, and underscores the important role of MV in activation of the tumor microenvironment.
INTRODUCTION

B-CLL has been predominantly characterized as a clonal B-cell disorder where the defective apoptosis of CLL B-cells is ascribed not only to intrinsic defects of the neoplastic cells but also to extrinsic factors that influence their behavior in the tissue microenvironment. The issue of CLL heterogeneity and the exact reasons for the clinical variety of disease progression are unknown. One important factor associated with disease progression is unfavorable prognostic features which may influence apoptotic resistance in the CLL B-cell clone but could be related to the ability of the clone to manipulate the microenvironment to its advantage. Recent studies demonstrate the importance of communication between tumor cells and their microenvironment through shedding of membrane microvesicles which can fuse to nearby cells within their circulatory pathways.

Microvesicles (MV) are shed from the cell surface of normal healthy or malignant cells and can ‘hijack’ membrane components and engulf cytoplasmic contents from either type of cell. Shedding of membrane-derived MV is a physiological phenomenon that accompanies cell activation and growth. MV contain numerous proteins and lipids similar to those present in the membranes of the origination cells and this likely facilitates their integration into cells they come in contact with during circulation. The content of microvesicles and their impact on biological function is dependent upon the cell of origin. Thus, it is known that ovarian cancer microvesicles stimulate angiogenesis, and platelet-derived microvesicles promote tumor progression and metastasis of lung cancer cells. It is likely that a substantial percentage of the so-
called soluble receptors identified in biological fluids or molecules such as DNA or mRNA are in fact associated with circulating MV \(^7\text{-}^9\). Given the attributes of the circulating MV in terms of their ability to transfer their contents to resident tissue cells, we questioned; 1) whether CLL plasma contained MV, 2) what their nature was, and 3) if they could influence the bone marrow stromal cells known to have close interactions that lead to both enhanced spontaneous and drug induced resistance of the CLL B-cells.

**METHODS**

**Isolation of microvesicles from CLL plasma and cell culture.** MV were isolated as previously described \(^10\) with minor modifications, from plasma of untreated CLL patients (n=60) or normal, healthy human subjects (n=5); each patient gave written informed consent from each subject, according to the Declaration of Helsinki, to the Mayo Clinic Institutional Review Board, which approved this study. The plasma samples were made free of platelets and cellular debris by centrifuging at 2500 x g for 20 min (repeated two more times). “Platelet-free plasma” was then centrifuged at 16000 x g for 1 hour in 4°C to precipitate MV. After wash in PBS, MV were resuspended in PBS and stored in 4°C for characterization.

The normal bone marrow stromal cell line (HS-5) and primary CLL B cells were cultured in appropriate growth media \(^11\). Primary BMSC were isolated from the bone biopsy materials and maintained *in vitro* as we have previously described \(^12\). For the MV stimulation experiments, serum-starved BMSC were stimulated with 30 μg/ml of MV for various periods of time as indicated and used for subsequent experiments. Conditioned
media were analyzed for cytokines using appropriate ELISA kits from R&D Systems (Minneapolis, MN).

**Reagents.** All the antibodies and inhibitors were purchased from Cell Signaling Technologies (Beverly, MA) with the exception of antibodies to VEGF (Santa Cruz Biotech, Santa Cruz, CA), VEGF<sub>165b</sub>, phospho-Axl (R&D Systems, Minneapolis, MN), c-myc, phycoerythrocyanin (PE)-conjugated antibodies to CD61, CD31 and CD19, and fluorescence-labeled isothiocyanate (FITC)-conjugated annexin V (BD Biosciences, San Jose, CA). Src/Abl kinase inhibitor SKI-606 was purchased from ChemieTek (Indianapolis, IN) and 1 μm beads from Sigma (St. Louis, MO).

**Flow cytometric analysis.** MV were double stained with annexin V and antibody to the platelet/megakaryocyte marker CD61, B-lymphocyte marker CD19, CD31, CD52 or CD20 and analyzed by flow cytometry using 1 μm beads (Sigma) for size<sup>13</sup>. Thus, we termed “platelet-derived MV” based on the presence of platelet/megakaryocyte marker CD61 and “B-lymphocyte-derived” based on the presence of CD19 cell surface marker.

**Electron microscopy.** MV were mixed 1:1 with 1% phosphotungstic acid (PTA) and adsorbed directly onto parlodium-coated 300 mesh copper grids. Following 3 brief washes with water, a layer of 1% PTA was added to the grid and allowed to dry prior to microscopy. Micrographs were acquired using a Technai G<sup>2</sup> 12 Transmission Electron Microscope (FEI, Inc., Hillsboro, OR), equipped with an AMT CCD camera system (Advanced Microscopy Techniques, Danvers, MA).
Labeling of MV with PKH67 and confocal microscopy. MV were labeled with a PKH67 green fluorescent labeling kit (Sigma) following the manufacturer's instructions. BMSC were incubated with the PKH67-labeled MV for the indicated time. Cells were washed, counter-stained with DAPI [(VectorShield), Vector Labs, Burlingame, CA] and subjected to confocal microscopy (Zeiss LSM 510 confocal Laser-scanning microscope, Munich, Germany). For staining with β-catenin, serum-starved CLL-BMSC were stimulated with MV for 48 hour and immunostained with a specific antibody to β-catenin, followed by Alexa-555 conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR). Cells were counter-stained with DAPI and visualized under confocal microscope.

Immunoblotting. Cells were lysed directly in SDS-Laemmli sample buffer and subjected to Western blot analyses as described previously 14.

Phospho-receptor tyrosine kinase antibody array. Equal amounts of cell lysates prepared as described 14 from primary CLL-BMSC stimulated with MV or left untreated for 24 hours, were added to the human phospho-receptor tyrosine kinase antibody arrays (R&D systems), following the manufacturer's instructions. The arrays were scanned and analyzed by densitometry. Relative pixel density was calculated and represented as fold activation.

Statistics. Statistical analysis was performed using two-tailed t-test.
RESULTS

Characterization of the plasma microvesicles in CLL

We have isolated MV from the plasma of previously untreated CLL patients in various disease stages (Rai 0-IV) as well as normal healthy human subjects. Flow cytometric analyses demonstrated that the majority of MV isolated from CLL plasma were positive for annexin V-binding (typically, >90%; Figure 1A) and sizes were within 1.0 μm, typical characteristics of MV, based on forward/side scatter using 1.0 μm standard beads. These were heterogeneous, membrane-encapsulated small vesicles ranging in size from 0.1-1.0 μm as revealed under electron microscopy (Figure 1B) also typical features of MV. While significantly higher levels (P < 0.006) of MV were demonstrable in the majority of CLL plasma (Figure 1C), we could not find a significant association of MV levels with disease stage or other prognostic factors (data not shown). These initial results revealed that CLL patients have increased levels of MV in their circulation and that they are typical of MV with heterogeneous size and are positive for annexin V-binding.

In normal plasma, the majority of MV have been shown to originate from platelets (80%) while the remainder come from endothelium (10%) and leukocytes (10%). To this end, while we observed a pattern of detecting more platelet-derived (CD61+, a platelet/megakaryocyte antigen) MV (~84%) in the earliest stage (Rai 0), a wide range of CD61 negative-bearing MV were demonstrable in the plasma of intermediate stage (Rai I/II) of CLL (Figure 1D). MV from more advanced stage (Rai III/IV) displayed an
average CD61+ level of ~50% (Figure 1D). Importantly, we detected relatively high levels (P = 0.068) of CD19+-MV in intermediate and significantly higher levels (P = 0.0004) in advanced stages of the disease as compared to early stage (Figure 1E). Since the major source of CD19 antigen is the B-lymphocyte, and the overwhelming B-cell population in advanced stage CLL is the leukemic B-cell which expresses CD19 membrane antigen, we assume that the source of the CD19+ MV is the leukemic B-cells (CD19+ MV are also referred to as “B-lymphocyte derived” in our text). However, we did not observe any clear association between total absolute lymphocyte counts and the levels of plasma MV (data not shown) suggesting the generation of leukemic cell-derived MV may depend more on the disease stage as determined by using the Rai staging system 17 which incorporates lymph node and spleen involvement with CLL rather than total blood lymphocyte counts. In total, these observations suggested a possible link between MV phenotypes and the progression of disease.

Microvesicles can integrate into leukemic B-cells and bone marrow stromal cells. Microvesicles are able to transfer various components of their contents into the membranes of target cells triggering a variety of biological responses 2. We first examined whether CLL-MV could transfer MV-associated surface proteins into target cells. Interestingly, we detected CD61 surface marker on the leukemic B-cells following incubation with CD61+-MV, suggesting transfer of CD61 from MV into the CLL B-cells as these leukemic cells do not express CD61 (Figure 2A). To further examine if MV are internalized by target cells, we incubated BMSC with the fluorescent dye PKH67-labeled MV. Binding of PKH67-MV to the BMSC surface was detected within an hour and they
were internalized within 1-2 hours (Figure 2B). Increased binding and internalization of more PKH67-MV into the target cells was detected with time (Figure 2B). In addition, we detected CD31 in MV-exposed BMSC delivered by the MV (Figure 2C) as BMSC do not express CD31\(^{18}\). These results suggest that the plasma CLL-MV possess the ability to bind and internalize into various target cells and transfer their contents to them.

**Microvesicles activate bone marrow stromal cells.**

Next, we interrogated the functional implication of MV-BMSC interaction because of the well recognized nurturing interaction between stromal cells and leukemic CLL B cells as well as the finding that these MV can transfer material to the BMSC. To address this, the normal human BMSC line (HS-5)\(^{19}\) was analyzed for the activation of various survival pathways following incubation with CLL-MV. Although we could not detect any activation of Erk1/2, NF-\(\kappa\)B or STAT3 (data not shown), a 30 min exposure to MV resulted in AKT phosphorylation at variable degrees (2- to 8-fold) in HS-5 cells (Figure 3A). We found primary BMSC isolated from CLL B-cell patients or normal human subjects responded to MV in a similar fashion (Figure 3B-D). MV-exposed primary BMSC maintained AKT in a sustained activated state for at least 24 h (Figure 3C&D). Thus, it appears that CLL-MV not only interact or are internalized into target cells; they can keep the AKT signaling pathway in a sustained state of activation in bone marrow stromal cells.

**Microvesicles activate the AKT/mTOR/p70S6K/HIF-1\(\alpha\) axis and induce VEGF expression in CLL-BMSC.**
Phosphatidylinositol 3-kinase (PI3K)/AKT is the central regulator of a number of signaling pathways involved in cell survival, proliferation, and angiogenesis. The mammalian target of rapamycin (mTOR)/p70S6K is activated by cytokines and growth factors through the activation of its upstream pathways, such as PI3K/AKT and mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase pathways (see model, Figure 4) and somatic mutations or deletions of the tumor suppressor PTEN. We examined whether the mTOR/p70S6K-pathway was activated in MV-exposed primary BMSC (Figure 4). A 3-fold activation of AKT was detected in MV-exposed CLL-BMSC, compared to MV unexposed cells. Treatment with wortmanin (PI3K-inhibitor) inhibited MV-mediated AKT-phosphorylation in BMSC, as expected. However, rapamycin (mTOR-inhibitor) treatment further increased the levels of AKT-phosphorylation in the cells incubated with MV alone or MV-unexposed cells (~5-fold) (Figure 4, top panel). This is consistent with a report that the effects on AKT-signaling by rapamycin are complicated by positive and negative feedback loops from mTOR to AKT in different cellular components of the tumor microenvironment. We also detected that MV-exposed CLL-BMSC displayed phosphorylation of p70S6K at the threonine residue (Thr-389) which was not inhibited by wortmanin suggesting possible cross-talk with other signaling pathways (Figure 4). Although activation of p70S6K involves phosphorylation of multiple serine and threonine residues, phosphorylation of Thr-389 by mTOR is critical for p70S6K activation and serves as a marker for mTOR activity. Importantly, rapamycin completely inhibited MV-mediated activation of p70S6K and phosphorylation at all the serine residues (235/236/240/244) of its downstream target 40S ribosomal protein S6 (S6rp), which were not inhibited by
wortmanin (Figure 4). Similarly, we observed activation of AKT in normal BMSC when exposed to MV which was inhibited to basal levels by wortmanin (Figure 4). Rapamycin treatment further enhanced AKT activation in normal-BMSC similar to CLL-BMSC; however, in contrast to CLL-BMSC, wortmanin was able to inhibit AKT-mediated activation of the p70S6K and its downstream targets in normal-BMSC, while rapamycin completely inhibited p70S6K, as expected (Figure 4, right panels). These findings suggest that MV-mediated modulation of AKT intracellular signaling pathway in normal BMSC is different than CLL-BMSC which could reflect an inherent functional difference in the two types of BMSC.

To further examine this possibility, we examined Erk1/2 activation in both normal and CLL BMSC. Erk is known to activate mTOR through inhibition of tuberin, an essential component of the AKT/mTOR signaling pathway in embryonic kidney cells and induces p70S6K activation. We explored whether the p42/44 MAPK (Erk1/2) pathway was activated by MV in primary BMSC. We found sustained activation of Erk1/2 in MV-exposed CLL-BMSC from their basal levels, but not in normal BMSC, which remained unaffected following treatment with wortmanin (Figure 4). This finding may explain why activation of p70S6K was not inhibited in MV exposed CLL-BMSC as a result of AKT-inhibition by wortmanin but was in normal BMSC.

p70S6K regulates the translation of a group of mRNAs possessing a 5′-terminal oligopyrimidine tract (5′TOP), a stretch of 4-14 pyrimidines found at the extreme 5′ terminus of certain mRNAs through phosphorylation of the S6 protein of the 40S
ribosomal unit and are predicted to account for 15-20% of total cellular mRNA. 
p70S6K would upregulate general translation capacity of the cells via increased 
translation of 5’TOP mRNAs. The 5’ untranslated region of HIF-1α mRNA contains 
5’TOP tracts and the translation is dependent on the mTOR/p70S6K pathway in certain 
cellular contexts (22). Indeed, we detected increased levels of HIF-1α protein synthesis 
in MV-mediated p70S6K-activation in CLL-BMSC; however, only a subtle, if any, 
increase in HIF-1α protein was detected in normal-BMSC following exposure to MV 
under similar experimental conditions (Figure 4, bottom panels). Consistent with this 
observation, we found that wortmanin had no detectable effect on HIF-1α expression in 
CLL-BMSC. However, rapamycin inhibited HIF-1α translation close to the basal levels 
suggesting that activation of the mTOR/p70S6K pathway resulted in the production of 
more HIF-1α in CLL-BMSC even under normoxic conditions.

HIF-1α is considered the master regulator of VEGF (VEGF-A), a potent pro-angiogenic 
and survival factor for CLL B-cells. To examine the functionality of HIF-1α expression 
in CLL-BMSC under normoxia, we measured the total VEGF production in conditioned 
media of MV-exposed CLL-BMSC by ELISA. VEGF production was elevated in the 
conditioned media but was inhibited by rapamycin, although it was not found to 
completely inhibit VEGF levels (Figure 5A). This is probably due to the positive 
regulatory effects of activated-AKT on VEGF transcription through Sp1 which is 
independent of HIF-mediated transcription. Although we found about two-fold 
increase in total VEGF production in normal BMSC exposed to MV (Figure 5A, right 
panel), it was well below the basal level production of VEGF by the unstimulated CLL-
BMSC (Figure 5A, left panel). We believe this finding is important in CLL B disease progression in that the \textit{in vivo} programming of CLL-BMSC to produce more VEGF may result in making them more prone to subsequent MV-mediated regulation than normal BMSC. The exact reason why CLL-BMSC have inherent functional signaling differences than normal BMSC remains unclear but could be related to prior \textit{in vivo} MV exposure.

The gene for human VEGF is organized into eight exons\textsuperscript{31}. As a result of alternative splicing, at least four transcripts encoding mature monomeric VEGF containing 121, 165, 189 and 206 amino acid residues (VEGF\textsubscript{121}, VEGF\textsubscript{165}, VEGF\textsubscript{189} and VEGF\textsubscript{206}) have been detected. The secretion pattern of the four isoforms differs markedly. VEGF\textsubscript{121} and VEGF\textsubscript{165} are the diffusible proteins secreted by various normal and transformed cells where VEGF\textsubscript{165} is the predominant secreted isoform. The VEGF\textsubscript{189} and VEGF\textsubscript{206}, on the other hand, are not freely secreted, but instead remain predominantly bound to the cell surface and/or extracellular matrix\textsuperscript{32}. Thus, to identify the predominant VEGF isoform(s) expressed in MV-exposed CLL BMSC, we analyzed the lysates of CLL BMSC exposed to MV with or without treatment with wortmanin or rapamycin by Western blot using an antibody to human VEGF (Figure 5B). We detected higher expression level of VEGF\textsubscript{165} in the MV-exposed CLL BMSC as compared to that in unexposed control BMSC cells. Importantly, MV-mediated upregulation of VEGF\textsubscript{165} was found to be the predominant isoform as compared to the VEGF\textsubscript{121} isoform which was in notably low levels in MV-exposed CLL BMSC (Figure 5B). Nonetheless, we could not detect VEGF\textsubscript{189} or VEGF\textsubscript{206} isoform in these cells under
similar experimental conditions. Together, these results suggest that MV induce expression predominantly of the VEGF\textsubscript{165} isoform in CLL BMSC.

To further evaluate the nature of the VEGF molecule secreted by the CLL-BMSC, we looked at two variants of VEGF\textsubscript{165}. VEGF\textsubscript{165} is alternatively spliced to form pro-angiogenic VEGF\textsubscript{165} and anti-angiogenic VEGF\textsubscript{165b}; the latter molecule has identical length but a differing C-terminus amino acid residue. The functional consequences of this altered C-terminus are that the VEGF\textsubscript{165b} homodimer competes with the pro-angiogenic VEGF\textsubscript{165} homodimer for binding to its receptors and thereby inhibits its function\textsuperscript{33}. Importantly, we could not detect any increase in VEGF\textsubscript{165b} production in the conditioned media of MV-exposed BMSC (both CLL and normal) from the basal levels (Figure 5C), suggesting that MV only induce production of the pro-angiogenic VEGF\textsubscript{165}.

**MV-mediated AKT activation in BMSC modulates the GSK3/\(\beta\)-catenin pathway.** Phosphorylation of GSK3\(\beta\) at Ser-9 residue by AKT results in the auto-inhibition of GSK3\(\beta\)\textsuperscript{34}. We found AKT-mediated inhibitory phosphorylation of GSK3\(\beta\) at Ser-9 in CLL-BMSC exposed to MV (Figure 6A). As expected, wortmanin, not rapamycin, inhibited AKT-mediated GSK3\(\beta\)-phosphorylation. GSK3\(\beta\) constitutively phosphorylates \(\beta\)-catenin at both serine and threonine residues in the amino-terminal region resulting in ubiquitination and subsequent proteasome-mediated degradation of the protein\textsuperscript{35}. We did not observe any significant accumulation of \(\beta\)-catenin in BMSC as a consequence of AKT-mediated inhibition of GSK3\(\beta\) (Figure 6A); instead, we found AKT-mediated direct phosphorylation of \(\beta\)-catenin (Ser-552) in MV exposed BMSC (Figure 6A) which was
reported to promote β-catenin transcriptional activity \(^{36}\). Although wortmanin inhibited AKT-mediated phosphorylation of β-catenin, rapamycin increased levels of β-catenin phosphorylation because of enhanced AKT-activation (Figure 6A). We also detected translocation of β-catenin to the nuclei of MV-exposed BMSC consistent with the phosphorylation of β-catenin at Ser-552 and the inhibition of GSK3β (Figure 6B). Importantly, the downstream targets of β-catenin, cyclin D1 and c-myc, were upregulated in BMSC exposed to MV (Figure 6C); further suggesting β-catenin was transcriptionally active in MV-exposed BMSC. As cyclin D1 is the downstream target of both β-catenin and GSK3β pathways \(^{37}\), upregulation of cyclin D1 in MV-exposed BMSC could be a result of a combined effect from these two pathways. These results indicate that MV not only activate the AKT/mTOR/p70S6K/HIF-1α axis, they can potentially modulate the AKT/GSK3β or AKT/β-catenin signaling pathways in BMSC.

**MV activate receptor tyrosine kinase activity in BMSC.**

Finally, we interrogated whether MV interact with specific receptors to initiate and generate signaling pathways which in turn activate AKT. To address this, we analyzed MV-exposed CLL-BMSC for the activation of any receptor tyrosine kinase (RTK) using phospho-RTK antibody array blots. Interestingly, MV from different CLL patients displayed similar patterns of activating EGFR, PDGFRβ, EphA7, EphB4 with Axl-activation (~4- to 7.5-fold) being the highest in CLL-BMSC as compared to the unstimulated control (Figure 7A). Axl overexpression has been reported in several types of human cancers including colon, prostatic, thyroid, breast, gastric, renal and lung \(^{38}\). Activation of Axl upon binding its ligand Gas6 (growth arrest-specific gene)
results in activation of PI3K and has been implicated in regulating cell survival, proliferation and migration \(^{39}\). However, we did not detect any significant expression of Gas6 in the MV-exposed BMSC (data not shown). Recently, it has been shown that Axl is a target of the Src/Abl inhibitor SKI-606, a 3-quinolinecarbonitrile compound \(^{40}\). We explored the possibility of whether MV-mediated Axl-activation was an upstream event responsible for the activation of AKT in BMSC using the inhibitor SKI-606. We found reduction of MV-mediated Axl-phosphorylation levels by SKI-606 with concomitant inhibition of AKT-phosphorylation in BMSC (Figure 7B), suggesting Axl-activation by MV may result in activation of AKT. We did not observe any significant expression of Gas6 in the BMSC lysates with or without stimulation by MV (data not shown). In contrast to a previous report \(^{41}\), use of anti-VEGF antibody did not exhibit any effect on Axl-phosphorylation in MV-stimulated BMSC, thus raising the possibility that MV delivered a constitutively active Axl directly to the BMSC. We detected low levels of phosphorylated Axl in MV isolated from early stage/low risk (Rai 0) and higher levels from Rai intermediate stages I/II (Figure 7C). These latter findings suggest that the phosphorylated Axl is likely to be delivered to BMSC by MV and seems to be one of the upstream events of AKT activation. Our preliminary work on CLL B-cells does in fact show easily detectable constitutively phosphorylated Axl receptor by immunoblot adding to the possibility that B cell derived MV in CLL plasma may contain Axl (Figure S1).

**Discussion**

In this study, we found elevated levels of plasma MV that appear to be predominantly platelet derived in early stage disease but with progression of disease become more B-
lymphocyte derived MV. These MV appear to have potent functional capacity in terms of modulating stromal cells. Thus, we have detected a very complex array of altered signaling pathways of the MV targeted BMSC. These results indicate that MV not only activate the AKT/mTOR/p70S6K/HIF-1α axis but can modulate the AKT/GSK3β or AKT/β-catenin signaling pathways in bone marrow stromal cells. Figure S2 is our attempt to summarize these events detailing more of our findings in this model. Prior work has shown that interaction of MV with target cells initiates various biological responses depending on the nature of this interaction. When MV fuse to their target cells, they can transfer important membrane components such as receptors and ligands (Figure S2B). Thus, microvesicle-mediated transfer of the truncated epidermal growth factor receptor (EGFRvIII) into U373 glioma cells resulted in a consistent increase in Erk1/2 phosphorylation. In our studies, CLL-MV mediated transfer of constitutively active Axl into BMSC could potentially alter normal cellular function by triggering/reprogramming other signaling cascades which may modulate the tumor microenvironment in favor of disease progression. For example, extracellular VEGF produced by the MV-stimulated BMSC may bind and activate PDGF-receptors [as reported earlier] on MV-exposed or -unexposed neighboring BMSC or endothelial cells in order to keep AKT in a state of activation (Figure S2C). An interesting and important finding was that we detected increased levels of phosphorylated Axl in CLL-MV from higher stages, which suggests the critical involvement of MV in the disease process. Thus, continuous and systemic circulation of MV with interaction via fusion to the BMSC membrane and subsequent transfer of critical signaling moieties to these stromal cells in various tissue sites is likely to favor disease progression.
The consequence of the CLL-MV interaction with BMSC is that certain key regulators of VEGF production are increased in these cells including HIF-1α. Angiogenesis likely plays an important role in the pathogenesis of CLL due to two known phenomenon: (i) increased neovascularization in the bone marrow and extramedullary tissues and (ii) the VEGF-based autocrine pathways for the accumulation of leukemic B cells. Interestingly, both the microvessel density and hotspot density in bone marrow trephine biopsy sections correlate positively with the clinical stage of the CLL patients. We found that CLL-BMSC are capable of producing more VEGF than their normal counterparts, and MV preferentially induce production of more VEGF from CLL-BMSC which may modulate the CLL-microenvironment in favor of CLL survival and resistance to chemotherapy. The study of VEGF secretion here has revealed important differences between the CLL and healthy control sources of BMSC, and we feel these findings imply that there are either some endogenous differences between normal and CLL BMSC or these cells have acquired modified signaling pathways that at least relate to VEGF production. Since we see heavily sustained activation of AKT in BMSC post MV exposure, we postulate that in vivo exposure to increased levels of MV may ultimately result in reprogramming of the BMSC with these qualities, and this change may in turn facilitate CLL disease progression.

MV from CLL plasma may also influence disease progression or drug resistance in other ways. Elevated levels of soluble CD52 (alemtuzumab-target) and CD20 (rituximab-target) have been previously reported in CLL plasma. Interestingly, we
detected that CD52 molecules were bound to the CLL-MV and the levels were increased as the disease progressed (Figure S3A). Although low, CD20 molecules were also detected on circulating CLL-MV (Figure S3B). These findings suggest that soluble CD52 and CD20 molecules, known to be present in CLL plasma, are likely to be microvesicle-bound. Thus, accumulation of CD52 and CD20 expressing MV in CLL circulation may potentially bind the specific monoclonal antibodies and contribute to the process of drug resistance to alemtuzumab- and rituximab-based therapies, respectively.

In summary, microvesicles, regardless of tumor types, can influence the tumor-microenvironment in favor of disease progression. Our studies have strongly implicated MV in the modulation of human stromal cells in B-CLL in particular with respect to VEGF production. We have also found that since MV express target receptors for two commonly used antibodies for treatment of CLL these MV may blunt treatment efficacy. Importantly, we have uncovered several unique aspects of the MV in terms of their inherent ability to modify the stromal cell environment that we believe can be exploited to both better understand disease progression in this currently incurable disease and also suggest novel therapies for these patients.

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AUTHOR CONTRIBUTIONS
AKG, designed, performed the experiments, analyzed the data and wrote the paper
CS, TK, WD: performed the experiments
DM and NEK: designed, analyzed the data and edited the paper

CONFLICT OF INTEREST DISCLOSURES: The authors declare no potential conflict of interest.

REFERENCES


FIGURE LEGENDS

**Figure 1. Identification and characterization of microvesicles.**  
A. Microvesicles (MV) were identified by evaluating their ability to bind annexin V by flow cytometry. Annexin-positivity is shown by histogram from two representative MV preparations from CLL plasma.  
B. Heterogeneity and sizes of the MV were determined by electron microscopy following negative membrane staining with phosphotungstic acid (magnification level is indicated by the horizontal bar).  
C. Plasma levels of MV were determined by measuring the total protein content and presented as μg of MV/ml of plasma [isolated from CLL patients (n=58) or normal human subjects (n=5)].  
D. & E. MV isolated from CLL patients at various disease stages as indicated were phenotypically characterized using CD61 for “platelet-derived” or CD19 for “B-lymphocyte-derived” cell surface marker by flow cytometry. Results from individual patients are presented with mean values.

**Figure 2. Microvesicles can integrate into various target cells.**  
A. Freshly isolated primary CLL B cells were incubated with MV isolated from different CLL patients for 48 hour. Transfer of CD61 marker from MV to CLL B cells was analyzed by flow cytometry.  
B. Purified microvesicles were labeled with the membrane dye PKH67 (green) and incubated with CLL-BMSC for the indicated periods. Binding and internalization of microvesicles into BMSC was visualized after staining with DAPI (nuclear stain) under confocal microscope (magnification level is indicated by the horizontal bar).  
C. Microvesicle-mediated transfer of message into BMSC was
examined by Western blot following a 24-hour incubation using an antibody to CD31. Expression of CD31 on microvesicles was verified by flow cytometry.

**Figure 3. Microvesicles activate human bone marrow stromal cells.** A. Microvesicles isolated from different CLL patients’ plasma exhibiting variable degrees of AKT activation in HS-5 cell line as determined by densitometric analysis of the results from Western blot. B-D. Microvesicles activate primary BMSC isolated from CLL or normal human subjects as analyzed by Western blot. A sustained level of AKT activation is detected at least up to 24 hours following incubation with microvesicles.

**Figure 4. Microvesicles activate the AKT/p70S6K/HIF-1α signaling axis in BMSC.** CLL- or normal-BMSC were exposed to microvesicles with or without presence of wortmanin or rapamycin for 24-hour. BMSC lysates were analyzed for the activation of the AKT/p70S6K signaling pathway as shown by Western blots using specific antibodies. Expression of HIF-1α, a downstream target of AKT/p70S6K axis is also shown. Actin was used as loading control. Microvesicle-mediated modulation of the AKT/p70S6K/HIF-1α axis is shown for better understanding (right panel).

**Figure 5. Microvesicles induce expression of VEGF in CLL BMSC.** A. Production of total VEGF in the conditioned media of BMSC stimulated with microvesicles as described above was measured by ELISA and mean values are presented with standard deviations (pg/ml per 10^5 cells). The asteric indicates statistical significance (p <0.0001) compared to the unstimulated controls. B. CLL-BMSC were exposed to
microvesicles with or without presence of wortmanin or rapamycin for 24-hours. Cell lysates were analyzed for the expression of different isoforms of VEGF by Western blot using an antibody to human VEGF. Results indicate microvesicles induce expression predominantly of the VEGF\textsubscript{165} isoform in CLL-BMSC. Actin was used as loading control. C. Production of the anti-angiogenic isoform VEGF\textsubscript{165b} in the above mentioned (panel B) conditioned media of BMSC stimulated with microvesicles was measured by ELISA and mean values are presented (pg/ml per 10\textsuperscript{5} cells).

Figure 6. Microvesicles modulate GSK3\textbeta/β-catenin signaling in BMSC. A. The BMSC lysates stimulated with microvesicles, described in Fig. 4A, were analyzed for AKT-mediated phosphorylation of GSK3\textbeta and β-catenin at Ser-552 by Western blot using specific antibodies. Phosphorylation of AKT is also shown for comparison. B. Translocation of β-catenin was visualized in BMSC stimulated with microvesicles from CLL patients (MV1 and MV2) using a specific antibody to β-catenin and nuclear stain, DAPI under confocal microscope (magnification level is indicated by the horizontal bar). C. AKT-mediated phosphorylation of β-catenin at Ser-552 and GSK3\textbeta was further examined in BMSC stimulated with microvesicles for 24- or 48-hour by Western blotting. Expression of cyclin D1 and c-myc, downstream targets of GSK3\textbeta/β-catenin was shown in BMSC following stimulation with microvesicles (MV22 and MV23) as compared to the unstimulated controls in Western blot analysis.

Figure 7. Microvesicles activate receptor tyrosine kinases in BMSC. A. Activation of receptor tyrosine kinases (RTKs) in BMSC stimulated with microvesicles obtained
from CLL patients at Rai stages 0 (MV1), I (MV2) or II (MV3) was analyzed on human phospho-RTK antibody array blots and the level of activation compared with the unstimulated control BMSC. Activation of various RTKs was analyzed and presented as fold-activation by bar diagrams. B. Lysates from BMSC stimulated with microvesicles following treatment with the Axl-inhibitor, SKI-606 (SKI) or anti-VEGF neutralizing antibody (NEB) or left unstimulated or untreated were examined for the phosphorylation of AKT by Western blot. Treatment with SKI-606 showed substantial inhibition of MV-mediated activation of AKT; however, anti-VEGF antibody did not show any effect. Phosphorylation of Axl is also shown for comparison. Actin was used as loading control. C. Microvesicles obtained from CLL patients at various Rai stages (0, I, II) were analyzed for the presence of constitutively active Axl by Western blot using an a phospho-specific antibody to Axl. Actin was used as loading control.
Figure 1

A

C.D.R. MV1

Unstained

Stained

Counts

Annexin FITC

C.D.R. MV2

Unstained

Stained

B

Electron Micrograph of MV

C

P<0.006

Pilanna MV level (µg/ml)

Normal
n=5

CLL B
n=58

D

CD51+ Microvesicles (%)

P=0.0007

P<0.0001

Low
(Rai 0)
n=14

Inter
(Rai I/II)
n=33

High
(Rai III/IV)
n=11

E

CD18+ Microvesicles (%)

P=0.068

P=0.0004

Low
(Rai 0)
n=13

Inter
(Rai I/II)
n=35

High
(Rai III/IV)
n=12
Figure 5

A

CLL B BMSC

Total VEGF expression (pg/ml)

MV-PL1  MV-PL2  MV-PL3

Normal BMSC

Total VEGF expression (pg/ml)

MV-PL4  MV-PL5

B

CLL BMSC

Nil  MV-PL6  MV-PL6+Wort  MV-PL6+Rap

VEGF

VEGF

Actin

C

CLL BMSC

VEGF\textsubscript{185} expression (pg/ml)

MV-PL1  MV-PL2  MV-PL3

Normal BMSC

VEGF\textsubscript{121} expression (pg/ml)

MV-PL4  MV-PL5
Circulating microvesicles in B cell chronic lymphocytic leukemia can stimulate marrow stromal cells: implications for disease progression

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