Microenvironment and Immunology

Fusion-Derived Epithelial Cancer Cells Express Hematopoietic Markers and Contribute to Stem Cell and Migratory Phenotype in Ovarian Carcinoma

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Abstract

For a long time, the external milieu of cancer cells was considered to be of secondary importance when compared with its intrinsic properties. That has changed now as the microenvironment is considered to be a major contributing factor toward the progression of tumor. In this study, we show that in human and mouse epithelial ovarian carcinoma and mouse lung carcinoma, the interaction between tumor-infiltrating hematopoietic cells and epithelial cancer cells results in their fusion. Intriguingly, even after the fusion event, cancer cells retain the expression of the pan-hematopoietic marker (CD45) and various markers of hematopoietic lineage, including those of hematopoietic stem cells, indicating that the hematopoietic genome is not completely reprogrammed. This observation may have implications on the bone marrow contribution to the cancer stem cell population. Interestingly, it was seen that in both cancer models, the expression of chemokine receptor CXCR4 was largely contributed to by the fused compartment of cancer cells. We hypothesize that the superior migratory potential gained by the cancer cells due to the fusion helps in its dissemination to various secondary organs upon activation of the CXCR4/CXCL12 axis. We are the first to report the presence of a hematopoietic cancer compartment, which contributes to stem cell markers and CXCR4 in epithelial carcinoma. This finding has repercussions on CXCR4-based therapeutics and opens new avenues in discovering novel molecular targets against fusion and metastasis. Cancer Res; 73(17); 5360–70. ©2013 AACR.

Introduction

Tumor is a conglomeration of various subpopulations like cancer stem cells (CSC), metastatic CSCs, proinvasive cells, etc., but how cancer cells achieve this heterogeneity is not deciphered completely. Apart from the intrinsic genetic factors, it is now recognized that the tumor microenvironment has a pivotal role in determining the events in carcinogenesis and metastasis (1–4). All solid tumors with or without chronic inflammation as a risk factor have a significant component of bone marrow-derived cells (BMDC; ref. 5) and various groups have tried to dissect their involvement in tumor progression. Chemical carcinogenesis of mouse BMDCs has been shown to give rise to epithelial, neural, muscular, endothelial, and fibroblastic tumors (6). Similar observation of donor-derived solid tumors has been made in patients who have undergone bone marrow transplantation (BMT; ref. 7). BMDCs are linked to the origin of gastric cancer resulting from chronic inflammation (8) and the report hypothesizes bone marrow-derived origin of CSCs, which has not been conclusively proved so far. All these studies implicate transdifferentiation of hematopoietic cells to an epithelial lineage. Moreover, a strong inflammatory response precedes the transformation, implying a void in knowledge on the course of events in tumors for which inflammation is not a risk factor or in patients who do not undergo BMT. The other mechanism by which BMDCs interact with cancer cells to alter their property is fusion. In vitro fusion of cancer cells with macrophages renders them with superior metastatic potential (9). In vivo cell fusion of circulating BMDCs with normal and transformed intestinal epithelia was shown in a mouse model (10). Subsequently, the same group proved that fusion imparts macrophage-specific properties such as invasiveness, immune evasion, etc., to the emerging hybrids in irradiated intestinal epithelia (11). Although fusion has been implicated in cancer, there are only a few reports that support its incidence and contribution to tumor progression. The hybrids resulting from hematopoietic–cancer cell fusion in an in vivo tumor model has not been characterized so far in terms of its migratory properties or CSC phenotype. We have tried to address these unanswered questions in our study. We report the presence of a hematopoietic-cancer cell fusion in metastasized human epithelial ovarian carcinoma (EOC), primary and metastasized mouse EOC, and primary Lewis lung carcinoma (LLC). The pathogenesis of EOC involves transcellular metastasis with formation of ascites, which makes it an
apt model to study the interaction between inflammatory cells and cancer cells (12). We have also studied the BMDC involvement in LLC model, which undergoes hematogenous and lymphatic metastasis (13). We address questions pertaining to the mechanism responsible for the existence of epithelial cancer cells expressing hematopoietic markers, their relevance in terms of the CSC hypothesis, and the tumor migration potential. We predict that active recruitment of hematopoietic cells to the tumor site due to the normal course of progression or proinflammatory conditions like irradiation could promote cancer cell–hematopoietic cell fusion, which may be conducive for the dissemination of cancer cells.

Materials and Methods

Cell lines and culture

ID8 (MOSSEC) cells (kind gift from Dr. Katherine Roby, University of Kansas Medical Center, Kansas City, KS) were transduced with GFP-expressing lentiviral particles for ID8-GFP and red fluorescent protein (RFP)-expressing retroviral particles for ID8-RFP cells. Similarly, LLC cells (American Type Culture Collection) were transduced with retroviral RFP particles for LLC–RFP cells (Supplementary Materials and Methods). Transduced cells in all cases were sorted using FACSariaIII (BD Biosciences). ID8-GFP and ID8-RFP were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 4% fetal calf serum (FCS), 1× insulin–transferrin–selenium (ITS), and LLC–RFP in DMEM with 10% FCS at 37°C in a humidified 5% CO2 incubator.

Human ovarian cancer cells

Ascitic fluid of cytopathologically confirmed serous EOC cases were obtained from the cytopathology laboratory of the All India Institute of Medical Sciences (New Delhi, India). Institute ethical clearance was obtained for using only residual ascitic fluid after routine processing and diagnosis. The samples were processed as per the approved procedures of the Human Ethics Committee at National Institute of Immunology (NIL, New Delhi, India). The mononuclear cells were isolated from the ascites by density gradient centrifugation using Histopaque (Sigma Aldrich).

Mouse ovarian cancer model

Orthotopic EOC was created in 6 to 8 weeks old syngeneic C57BL/6j strain of mice by injecting 1×10³ ID8-GFP cells under the ovarian bursa using a microsyringe (Kloehn) followed by survival surgery. For experiments not involving the primary tumor, 5×10⁶ ID8-GFP cells in 0.2 mL DMEM were injected intraperitoneally in the same strain of mice. Mice were obtained from the Jackson Laboratory and maintained in the experimental animal facility of the institute. All experiments using mice were carried out as per procedures approved by the Institutional Animal Ethics Committee at NIL.

Ex-vivo fusion

An inflammatory reaction was induced in 6 to 8 weeks old C57BL/6j mice by injecting 1.5 mL of 4% sodium thioglycolate (Sigma) through intraperitoneal route. Ascites were collected 72 hours after injection. The inflammatory cells of ascites and the ID8-GFP cells were mixed at 5:1 ratio and cultured for 48 hours in DMEM with 4% FCS and 1×ITS at 37°C in 5% CO2 incubator.

In vitro migration assay

In vitro migration assay was carried out following the protocol described in Supplementary Materials and Methods. CXCL12 was used as the chemoattractant to compare the migratory potential of sorted GFP+CD45+ and GFP+CD45− ID8 cells from the ascites.

Analysis of cell fusion

DNA content (ploidy). DNA content of the various subpopulations of primary tumor, ascites, and cultured ID8 cells was analyzed. The cells were resuspended (1 million cells/mL) in 2% FCS containing DMEM with 10 mmol/L HEPES and 5 µg/mL Hoechst 33342 (Sigma). They were incubated at 37°C for 90 minutes followed by staining with 1:200 anti-mouse CD45-APC (eBiosciences) at 4°C. To gate out dead cells, 1 µg/mL propidium iodide (Sigma) was added and doublets were discriminated using Hoechst Blue-width parameter.

RFP/GFP dual reporter models. For ovarian carcinoma model, ID8-RFP cells (5×10⁶/mouse) in 0.2 mL DMEM were injected intraperitoneally in 6- to 8-week-old GFP-expressing C57BL/6j [C57BL/6-Tg(UBCGFP)30Scha/J] transgenic mice. For lung carcinoma model, LLC–RFP cells (1×10⁶/mouse) in DMEM were injected subcutaneously in the same strain of mice. The fused cells were detected on the basis of their expression of RFP and GFP by flow cytometry and microscopy.

FISH. ID8-GFP cells were injected intraperitoneally in sex mismatched bone marrow chimera formed by reconstituting irradiated female mice with male bone marrow cells (BMC; Supplementary Materials and Methods). FISH of mouse X and Y chromosome was conducted in cells from ascites as described in Supplementary Materials and Methods.

Absolute quantification of Sry gene. Absolute quantification of the Sry gene was conducted in sorted GFP+CD45.2+ and GFP+CD45.2− ID8 cells from the ascites of chimeric mice by real-time qPCR (Eppendorf Realplex) using SYBR Green (Applied Biosystems) chemistry. Standards for Sry gene were prepared by mixing known numbers of ID8(XX) and male BMC (XY) at different dilutions corresponding to percentage fusion (Supplementary Materials and Methods). Genomic DNA (gDNA) was isolated with kit (Fisher Scientific) from the standard cell mixtures and sorted cells. The same quantity of gDNA for each template was used for the PCR of Sry gene (Supplementary Materials and Methods). The Ct values of sorted samples were fit in the standard curve generated by the Realplex software (Eppendorf) and their percentage fusion was determined.

Relative quantitative real-time PCR

For relative quantitative real-time PCR was carried out for genes of interest in various template cDNA samples by SYBR Green chemistry and fold change was calculated by 2−ΔΔCt method. The protocols for RNA isolation, cDNA synthesis, primer sequences, and PCR reaction parameters are provided in Supplementary Materials and Methods.
Flow cytometry and cell sorting
Details of the staining protocol and antibodies used for flow cytometry analysis are provided in Supplementary Materials and Methods. For cell sorting, the erythrocytes were removed by depleting Ter119+ cells by MACS (Miltenyi Biotech). Sorting of GFP+, CD45+ GFP+, and CD45− GFP+ was done with a 100 µm nozzle. Doublets were discriminated using pulse-width parameter. For improving sort purity of the CD45+ fraction, the cells were re-sorted in the same mode. All the analyses and sorting were conducted on FACS AriaIII (BD Biosciences).

Immunocytochemistry and immunohistochemistry
The standard staining protocol for immunocytochemistry of cytopsin of cells from ascites and immunohistochemistry of paraffin-embedded primary tumor tissue was followed as described in Supplementary Materials and Methods. Leica SP5 II confocal laser-scanning microscope with Plan-Apochromat 63×/1.4 oil objective was used for imaging and LAS/AF Lite software for analysis. The images of tissue sections were taken as Z-stack series.

Statistical analysis
Results of multiple experiments were reported as the mean ± SEM. Student t test was carried out to calculate the significance between 2 means.

Results
Hemato-epithelial cellular compartment contributes to the expression of markers of stemness and migration in human EOC
The cancer cells in ascites from 4 patients with EOC were identified on the basis of established biomarkers CA125 and EpCAM (14). Intriguingly, irrespective of the biomarker chosen, 16.4% to 23.9% of the cancer cells expressed the pan-hematopoietic marker CD45 (Fig. 1A). The presence of this subpopulation was further validated by confocal microscopy (Fig. 1B) and we would hence refer to it as the "hemato-epithelial" cells as they express both hematopoietic and epithelial cancer biomarkers. As a significant number of cancer cells were CD45-, we investigated its association with other hematopoietic stem cell (HSC) antigens. In ovarian carcinoma, CD44+CD117+Lin− (15) and CD133+ (16) are established CSC phenotypes. Our results suggest that within CD44+CD117+ phenotype, a major fraction of cancer cells expressed CD45 antigen, whereas in the other 2 phenotypes analyzed (CD133 or CD34), the expression of CD45 was limited to a smaller fraction of cells (Fig. 1C, Supplementary Fig. S1). These results may have direct bearings on the CSC hypothesis and phenotype in ovarian carcinoma. Similarly, the CD45+ cancer cells also contributed to the expression of promigratory marker CXCR4 (17), indicating that the hematopoietic nature could improve their potential to migrate (Fig. 1C, Supplementary Fig. S1). As previous reports have implicated both differentiation (8, 18, 19) and fusion (10, 11) in the bone marrow origin of epithelial cancer, it was important to decipher how and why certain ovarian cancer cells express CD45.

A subpopulation of ID8 cells expresses CD45 during the course of tumor progression
As it was not possible to address mechanistic questions of BMDC involvement in human ovarian cancer progression, a mouse model was chosen to dissect the occurrence of the above phenomenon. Spontaneously transformed surface epithelial cells of mouse ovary (ID8; ref. 20) were used to generate tumors in syngeneic mice, because it was important to closely mimic the patient scenario with an intact competent immune system. When ID8-GFP cells were injected intraperitoneally, they formed metastatic tumor nodules concomitant with the development of ascitic fluid in the peritoneal cavity, which resembled high-grade serous EOC in humans. The GFP+ cancer cells isolated from the ascites (Supplementary Fig. S2A) displayed the transcription of CD45, which was not seen in the parent population used to generate the tumors (Fig. 2A). To understand whether the CD45 expression is restricted to the metastatic cells in the ascites or also found in the solid mouse ovarian tumor, an orthotopic tumor model was created (Supplementary Fig. S2B) and the primary tumor was isolated before ascites formation (Supplementary Fig. S2C). It was observed that the percentage of GFP+CD45− cells was significantly higher in the primary tumor when compared with the ascitic fluid (Fig. 2B). Confocal microscopy of the primary tumor showed that the morphology of GFP+CD45− cells was epithelial and not an artifact of intraepithelial lymphocytes. Similarly, microscopic examination revealed that the ascites consisted of both binucleated and mononucleated GFP+CD45− cells (Fig. 2C). The observation of a CD45-expressing cancer population in the ID8 mouse model was a recapitulation of the phenomenon seen in human patients; hence, this model was further explored to understand the expression of hematopoietic phenotype in EOC.

CD45-expressing cancer cells are resultant of cancer cells fusing with hematopoietic cells recruited to the tumor site
When the DNA content of the digested primary tumor and the mononuclear cells in the ascites was analyzed, it was seen that most of the GFP+CD45− host-derived hematopoietic cells recruited to the tumor site were diploid (2n), the GFP+CD45+ cancer cells were tetraploid (4n), and majority of the GFP+CD45+ cells were hexaploid (6n), suggesting that the G0–G1 DNA content of the hemat-epithelial population was a sum of the diploid hematopoietic cells and tetraploid cancer cells (Fig. 3A; Supplementary Fig. S3A). To establish that this additive ploidy was a result of fusion between the hematopoietic cells and cancer cells, a dual reporter model of ID8-RFP injected in GFP-C57Bl/6J mice was adopted. It was observed that the ascites in such mice had a compartment of cancer cells that were hexaploid (6n); implying that the cancer cells preferentially fuse with hematopoietic cells. The presence of CD45 antigen on the surface of GFP+ "yellow" cells was confirmed by single plane confocal microscopy (Fig. 3B, i). Although we showed the presence of CD45 expressing fused cancer cells with the
RFP/GFP dual reporter model, it was not possible to detect those fusion hybrids whose hematopoietic genome had undergone complete nuclear reprogramming. To identify such cells, an alternate reporter system of sex mismatched bone marrow chimera was used, where ID8-GFP cells were injected intraperitoneally in female mice chimeric with male CD45.2 BMC. After tumor generation it was observed that the GFP⁺ cancer cells in the ascites now had a subpopulation of cells that expressed the CD45.2 isoform, indicating their bone marrow origin (Supplementary Fig. S3B). Interestingly, the percentage of hematopoietic cancer cells was significantly higher in chimeric mice when compared with normal tumor-bearing mice (Supplementary Fig. S3C). The cells in the ascites of these mice were probed for X and Y chromosome by FISH and it was observed that 4.37% of the nuclei counted (Fig. 3C and Supplementary Fig. S3D) had multiple X chromosomes and a Y chromosome, which corresponded to the percentage of GFP⁺CD45.2⁺ cells in the ascites. As the parent ID8 cells were near tetraploid, the number of X chromosomes in these cells was variable as previously reported (20). The presence of Poly X cells with Y chromosome in the ascites was probably due to the fusion of ID8 cells with male BMDC. However, to rule out the possibility of fusion of host hematopoietic (XX) cells with the donor-derived (XY) cells, the hematopoietic GFP⁺CD45.2⁺ population was sorted by fluorescence-activated cell sorting (FACS; Supplementary Fig. S3E). Furthermore, the GFP⁺CD45.2⁺ cells were also sorted to detect those cells that had probably undergone fusion but lost the expression of CD45. The gDNA of these two-sorted populations was analyzed for their percentage fusion by absolute quantification PCR of Sry gene. In comparison with the standards, it was observed that only 0.73%...
5.51% of the GFP genome was silenced after fusion. It was seen that 54.1% of the GFP cells in the ascites consisted of distinct subpopulations displaying HSC phenotype Sca-1 
CD45 
CD44 
Lin 
CD34 
s were Sry 
CD45.2 
CD45 A

Cancer cells express markers of various hematopoietic lineages, including those of stem cells after fusion

Apart from the cells of myeloid (Mac-1, Gr-1) and lymphoid (B220, CD90.2) lineages—a small but significant number of cells displaying HSC phenotype Sca-1 
 c-Kit 
 Lin 
 LSK were also recruited to the ascites during tumor progression. Interestingly, the CD45+GFP+ cells also expressed the markers Mac1, Gr1, B220, CD90.2, and LSK (Fig. A and Supplementary Fig. S4A) and the percentage expression was similar to the composition of the ascitic fluid. These results suggest that fused cancer cells were dominantly myeloid (~80%) and the hematopoietic genome of the hybrids was not completely silenced. The ID8-GFP cells in the ascites consisted of distinct subpopulations displaying stem cell phenotypes LSK, CD34+, and CD44+ CD117+ (Fig. B, i and Supplementary Fig. S4B). Interestingly, the parent ID8 cells grown in culture did not display these phenotypes (Supplementary Fig. S4C), strongly supporting the contribution of the microenvironment in the gain of these stem cell markers. When these populations were further analyzed, it was revealed that CD45+ cancer cells contributed largely to the LSK and CD34+ populations in the ascitic fluid, implying that fusion may have a role in maintaining stem cell-like phenotype in mouse ovarian carcinoma (Fig. B, ii). This finding was comparable with the observation in human patients; however, in this model, the CD34 and LSK populations were largely CD45- and the CD44+ CD117+ population was mostly CD45- unlike the human patients (Fig. C). The presence of inflammatory cells seemed to be important for fusion because they were constantly recruited to the tumor site throughout the course of tumorigenesis and the CD45-expressing ID8 cells
Significance of Hemato-Epithelial Ovarian Cancer Compartment

Figure 3. ID8 cells undergo fusion with BMDCs during tumor progression. A, DNA contents of the GFP+CD45+, GFP+CD45−, and GFP−CD45+ cells in the primary tumor and ascitic fluid were estimated by nuclear staining with Hoechst 33342 dye. The representative flow cytometry analyses of live cells show that majority of GFP+CD45+ population was hexaploid, which was additive of the diploid immune cells and tetraploid ID8-GFP cells. B, fusion in dual reporter model of ID8-RFP injected in GFP-C57Bl/6J mice. Flow cytometry analysis of the cells in the ascites show the presence of RFP+GFP+ cancer cells, which are mostly CD45+. CD45 expression in RFP+GFP+ cells is confirmed by single plane confocal microscopy (i). C, FISH of X and Y chromosome in ascites generated by ID8-GFP cells in sex mismatched bone marrow chimeric mouse. Cells of the ascitic fluid were analyzed for the presence of X (red) and Y (green) chromosome in the nuclei (blue). The results show the presence of a compartment of cells bearing multiple X chromosomes and a Y chromosome, indicating the occurrence of fusion. D, quantitative real-time PCR of Sry gene. Fusion was determined by absolute quantification of Sry gene in the gDNA of sorted GFP+CD45.2+ and GFP−CD45.2− cells from the ascites. Donor-derived Sry gene was almost absent in the GFP−CD45.2− compartment, whereas all the cells in the GFP+CD45.2+ fraction were fusion hybrids. Magnifications, ×630 and ×1,000; n = 3.

were observed even at very late stages of cancer when mice start displaying morbidity. To prove that interaction with the inflammatory cells was sufficient to cause fusion, ID8 cells were cocultured with sodium thioglycolate-induced intraperitoneal inflammatory cells. It was seen that 48 hours of culture, a small percentage of ID8 cells started expressing CD45 and most of this population too was hexaploid (Fig. 4C and Supplementary Fig. S4D), indicating that the mere presence of inflammatory cells with cancer cells was sufficient to cause their fusion.

Fused cancer cells maintain epithelial phenotype and gain promigratory property

Although the characterization of the fused population revealed the presence of a number of hematopoietic markers, it was imperative to determine whether they also expressed epithelial phenotype. Apart from the active transcription of GFP, it was seen that both the GFP+CD45+ and GFP−CD45− populations had a comparable expression status of epithelial markers cytokeratin 18 (CK18) and CK19 known to be present in circulating or noncirculating tumor cells but absent in
CD45 antigen. A subpopulation of cultured cancer cells express CD45 antibody, and which were mostly hexaploid in nature as seen by DNA content analysis. Fusion of ID8 and immune cells with ID8-GFP cells for 48 hours. A representative dot plot and histogram was recapitulated by coculturing sodium thioglycolate-induced ascites cells, including those of myeloid, lymphoid, and HSC lineages, are recruited to the ascitic fluid and the phenotypes of these cells are found to express in the fused compartment of GFP+ ID8 cancer cells via the hematogenous and lymphatic route was chosen. A dual reporter model of LLC-RFP cells injected subcutaneously in transgenic GFP-C57Bl/6 mice was used. As observed in mouse ovarian carcinoma, a small but significant percentage (7.9% ± 0.40%) of RFP+ LLC cells were also GFP+. Majority of these RFP+“GFP+” cells were CD45+, clearly showing that fusion occurs more commonly than previously thought (Fig. 6A, i). The presence of CD45 was observed on the surface of RFP+“GFP+” cells in enzymatically digested tumor tissue by single plane confocal microscopy (Fig. 6A, ii). Apart from CD45, the RFP+“GFP+” cells expressed markers of the myeloid, lymphoid, and HSC lineages (Fig. 6B and Supplementary Fig. S6A). Most of the CXCR4 expression in LLC was from the GFP+ compartment of cancer cells, implicating a potential role of this population in the dissemination of tumor cells via the hematogenous route (Fig. 6C). It was also observed that majority of hematopoietic cells (21). Similarly, Amhr2, which is exclusively expressed by ovarian tissue (22) and oncogenes c-Myc and β-catenin, was also found to be transcribed in both fused and unfused cancer compartments of the ascites (Fig. 5A). CK19 expression was also observed by single plane confocal microscopy in the GFP+“CD45+” cells (Supplementary Fig. S5A). To understand the role of fusion in the migratory potential of cancer cells, the expression of promigratory marker CXCR4 was analyzed. It was seen that in the fused cancer cells, the gene expression of promigratory receptor CXCR4 was drastically upregulated when compared with the unfused cancer cells. It is noteworthy that the parent ID8 cells used to generate the tumors did not express CXCR4 (Fig. 5B). The percentage expression of CXCR4 was similar in the GFP+ cancer cells in the primary tumor and ascitic fluid (data not shown). However, most of the CXCR4+ cancer cells in the ascites expressed CD45, whereas a lesser percentage of the CXCR4+ cells in the primary site were CD45+ (Fig. 5C). A significantly less percentage of infiltrating CD45+ cells were seen in the primary tumor in comparison with the ascites (Supplementary Fig. S5B, i and ii). In contrast with the primary tumor, the ascites also displayed a much higher percentage expression of CXCR4 within the recruited hematopoietic population (Supplementary Fig. S5B, i and iii). The CXCR4-expressing GFP+CD45+ cells were visualized by confocal microscopy in the primary periphery and ascites (Supplementary Fig. S5C). The migratory potential of the GFP+CD45+ cells toward a CXCL12 gradient was found to be superior (P = 0.0103) when compared with the GFP+CD45+ cells in an in vitro migration assay, indicating that the fused population was functionally promigratory in nature (Fig. 5D). To further dissect the significance of multilineage expression in fused cancer cells, the Mac-1+ Gr-1+ myeloid and the B220+CD90.2+ lymphoid populations were sorted from sodium thioglycolate-induced intraperitoneal inflammatory cells (Supplementary Fig. S5D, i). During isolation, it was seen that the myeloid cells had a significantly higher expression of CXCR4 when compared with the lymphoid cells (Supplementary Fig. S5D, ii). When the 2 inflammatory cells types were cocultured independently with ID8-GFP, it was observed that both kinds of immune cells had similar fusion efficiency with cancer cells. However, it was the myeloid-ID8 fused population that displayed superior CXCR4 expression (Fig. 5E, i and ii). The in vivo observation of multilineage fused cells imply that although cancer cells fuse with all the hematopoietic cells they interact with, it is the myeloid compartment that largely confers the promigratory CXCR4+ phenotype on the hybrids.

**Hemato-epithelial cells are not restricted to ovarian carcinoma**

To confirm that the phenomenon of hematopoietic cells fusing with cancer cells is not restricted to ovarian carcinoma, an alternate mouse LLC model that metastasizes via the hematogenous and lymphatic route was chosen. A dual reporter model of LLC-RFP cells injected subcutaneously in transgenic GFP-C57Bl/6 mice was used. As observed in mouse ovarian carcinoma, a small but significant percentage (7.9% ± 0.40%) of RFP+ LLC cells were also GFP+. Majority of these RFP+“GFP+” cells were CD45+, clearly showing that fusion occurs more commonly than previously thought (Fig. 6A, i). The presence of CD45 was observed on the surface of RFP+“GFP+” cells in enzymatically digested tumor tissue by single plane confocal microscopy (Fig. 6A, ii). Apart from CD45, the RFP+“GFP+” cells expressed markers of the myeloid, lymphoid, and HSC lineages (Fig. 6B and Supplementary Fig. S6A). Most of the CXCR4 expression in LLC was from the GFP+ compartment of cancer cells, implicating a potential role of this population in the dissemination of tumor cells via the hematogenous route (Fig. 6C). It was also observed that majority of
the CXCR4 expressing fused LLC cells displayed myeloid phenotype (Supplementary Fig. S6B).

Discussion

The contribution of BMDC to tumor epithelia is a debated area of research, with conflicting reports both on the presence of such cells and the mechanism responsible for their existence. One school of thought is based on the plastic nature of BMDC (6), which results in their transdifferentiation to cancer cells and the other is on the fusogenic ability of neoplasia and immune cells (23, 24). The key finding of the present study is the presence of pan-hematopoietic marker CD45 in a compartment of human and mouse EOC and LLC, which has not been shown so far in any report supporting either school of thought. Helicobacter felis infection–induced transformation involves the transdifferentiation of BMDCs to gastric cancer epithelia (8). However, both ovarian carcinoma and LLC are known to elicit a strong inflammatory response as a consequence of carcinogenesis and not vice versa. So, our finding supports the role of hematopoietic cells in tumor progression but not in the origin of cancer per se. In the ApcMin/+ mouse model (25), where intestinal carcinogenesis is followed by an inflammatory response, there are contradictory reports that attribute developmental mimicry (18) and cell–cell fusion (10) to the presence of bone marrow-derived adenomas. On the basis of dual reporter systems both in mouse EOC and LLC, we show incontrovertible evidence for the occurrence of hematopoietic cell–cancer cell fusion. Most of the previous studies on fusion in patients and mice have used models that are either irradiated (7, 10, 26, 27) or undergo surgical procedure like parabiosis (11) to achieve a system for defining the BMDCs. We observe the hemato-epithelial compartment of cancer cells
during the natural course of tumorigenesis without an initial whole body irradiation and BMT. Although we have also used the bone marrow chimera-based Ychr/GFP dual reporter system, it only substantiates our findings on fusion in the ID8 model and adds to the other experiments. Irradiation is known to cause inflammation and hyperproliferation with enhanced recruitment of BMDCs to the damaged tissue (28), which might be the reason why we observe a higher percentage of hematopoietic cancer cells in chimeric mice. None of the patients whose ascites were analyzed had undergone chemotherapy or irradiation, yet the presence of the hematopoietic tumor compartment strengthens the notion that bone marrow may be involved in conferring stem cell properties to the neoplastic cells. Our study implicates that all CD45− RFP+ GFP+ CD45− cancer cells as analyzed by flow cytometry. C, flow cytometry–based analysis of the primary tumor shows that GFP+ compartment of LLC–RFP contributes significantly to the CXCR4 expression. Magnification, ×630; scale bar, 10 μm. n = 3.

Figure 6. LLC also exhibits fusion and hematopoietic phenotype. A, fusion between LLC and hematopoietic cells in a dual reporter model of LLC–RFP cancer cells in GFP-C57B/6J mice. Flow cytometry analysis of enzymatically digested subcutaneous LLC–RFP tumors show a significant percentage of RFP+ GFP+ cells, which also express CD45+. Single plane confocal microscopy of cytospins of digested tumor tissue confirms the presence of RFP+ GFP+ CD45+ cells (i). B, hematopoietic markers, including those of myeloid, lymphoid, and HSC lineages, were expressed in the fused compartment of RFP+ GFP+ CD45+ cancer cells as analyzed by flow cytometry. C, flow cytometry–based analysis of the primary tumor shows that GFP+ compartment of LLC–RFP contributes significantly to the CXCR4 expression. Magnification, ×630; scale bar, 10 μm. n = 3.

LSK population in both ID8 and LLC tumor sites. The most important implication of this finding is the possible contribution of the infiltrating hematopoietic cells to the CSC phenotype. The coexpression of CD45 and CD44+ CD117+ in the human, and CD45 and LSK/CD34− in the mouse ascites tumor cells compartment strengthens the notion that bone marrow may be involved in conferring stem cell properties to the neoplastic cells. Our study implicates that all CD45− expressing ID8 and LLC cells are resultants of fusion and by extrapolation the stem cell marker expressing CD45− cancer population is also fusion derived. As these markers are gained over the course of tumor progression, they should ideally be referred to as metastatic CSC-like cells, which are needed for tumor progression but not initiation. HSC markers like CD34 (32) and CD133 (33) are also characterized as CSC markers in solid tumors (34, 35). It is possible that such CSCs are derived due to the fusion or without nuclear reprogramming. We show the fusion of cancer cells with both differentiated hematopoietic cells and HSCs. It is widely accepted that HSCs are inherently quiescent and long living. Thus, HSC–cancer cell fusion event may result in hybrids, which are stably maintained over a longer span of tumor progression.

Fusion between normal nonmalignant cell types is followed by nuclear reprogramming (36). The same is seen in a cancer-like state of irradiation-induced inflammation of intestinal
epithelia, where F4/80 expression is seen at very early stages of fusion but lost over the period of time (11). But in our study, we see the expression of not only CD45 but also of various myeloid and lymphoid markers in ID8 and LLC mouse models till advanced stages of cancer. The presence of epithelial phenotype in the fused ID8 cells and its retention of ovarian tissue-specific marker strongly suggest that even if nuclear reprogramming is occurring, it is incomplete and both cancer and hematopoietic genes are being transcribed extensively. It has been hypothesized previously that fusion can lend myeloid traits to cancer (37) and incomplete reprogramming observed by us implies the stable introduction of hematopoietic properties in the cancer genome. The gain of migratory property by the fused cells in terms of the expression of CXCR4 is the most clinically relevant finding from this study. On the basis of findings in irradiated intestinal epithelia, it has been speculated that cancer cells can become more metastatic upon fusion (11). Our study proves beyond any doubt that in vivo fusion with hematopoietic cells can make cancer cells more migratory. The periphery of tumor is usually considered as its invasive front (38) and the CXCR4 expressing fused ID8 cells observed in this region may be the cells on the verge of undergoing dissemination from the primary site. Our observation shows that the fused cells contributed significantly more to CXCR4 expression in the ascites when compared with the primary tumor. We attribute this to our finding that the percentage of hematopoietic component of the ascites is much higher than that in the primary tumor. Moreover, within the hematopoietic population also the CXCR4-expressing CD45+ cells are significantly more in the ascites when compared with the solid tumor. The proximity of cancer cells to immune cells in the interperitoneal suspension may further enhance the chances of fusion. These observations in ovarian carcinoma also suggest that fusion and concomitant CXCR4 expression in the ascites improves the prospects of cancer cells reseeding to form secondary metastases on specific organs producing CXCL12. However, in LLC, it was seen that even in the primary tumor most of the CXCR4-expressing cells were derived. Unlike transcelomic metastasis where the tumor cells remain in an intermediate inflammatory suspension, the CXCR4 expression here could be more significant for the migration of the cancer cells from the primary tumor to sites like the bone marrow by activation of CXCR4-CXCL12 axis (17). Fusion seems to be a phenomenon common to two different modes of metastasis. We have already showed that the presence of inflammatory cells in vitro with cancer cells is sufficient to cause their fusion. Although the interaction of ovarian cancer cells with inflammatory immune cells is more intimate in the ascites, there exists a robust relationship between cancer cells and hematopoietic cells in both forms of tumors. This might be the reason we observe fusion in two diverse metastatic modes. Similarly, CXCR4 has been described as a promigratory marker in both transcelomic (39) and hematogenous (40) metastasis. Thus, fusion and the resultant CXCR4 expression would enhance the migratory potential of cancer cells in both cases. Knockdown of CXCR4 has been shown to reduce the secondary tumor deposit in ovarian cancer (41) and inhibitors of CXCR4-CXCL12 axis have been targeted in many forms of cancer including breast (42) and multiple myeloma (43). We observe that a large majority of the CXCR4 expression both in EOC and lung cancer is derived from the fused compartment of cancer cells, which would mean that apart from inhibitors, antagonists, and knockdowns, one can also consider fusion as a plausible target to abrogate the expression of CXCR4.

With the present study, we implicate fusion as a key mechanism responsible for conferring enhanced migratory properties to cancer cells. We propose that these cells are better equipped for site-specific migration and the stem cell-like compartment in such a subpopulation may act as ‘seeds’ for dissemination of the primary tumor. Furthermore, exploration of these cells and the fusogenic molecules can provide us with novel targets for curbing tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Significance of Hemato-Epithelial Ovarian Cancer Compartment

References

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