CD24 Offers a Therapeutic Target for Control of Bladder Cancer Metastasis Based on a Requirement for Lung Colonization

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Abstract

Metastasis is lethal in most bladder cancer patients. Expression of CD24, a glycosyl phosphatidylinositol (GPI)-linked sialoglycoprotein and cancer stem cell marker, is associated with metastatic progression in multiple cancer types, yet the role of CD24 in this process remains unclear. While developing a murine model of human metastatic bladder cancer, we observed that tumor cell CD24 expression correlated with a propensity to metastasize to the lung. Our immunohistochemical evaluation of 60 paired primary and metastatic human bladder cancer samples revealed increased intensity ($P < 0.001$) and frequency ($P < 0.001$) of CD24 expression in metastases. To directly evaluate the role of CD24 in metastatic colonization, we manipulated CD24 expression in human bladder cancer cell lines using short hairpin RNA depletion, cDNA overexpression, and fluorescence-activated cell sorting selection. Although suppression of CD24 reduced acute tumor cell retention in the lungs of mice inoculated intravenously with cancer cells, this differential retention was no longer apparent after 24 hours, prompting us to evaluate the role of CD24 in lung colonization. Here, CD24 was found necessary for subsequent development of lung metastases. We next treated clinically detectable lung metastases in mice with anti-CD24 antibody and observed reduced tumor growth and prolonged survival. These findings suggest that CD24 is a lynchpin of metastatic progression and a promising therapeutic target for antimetastatic therapy. Cancer Res; 71(11); 3802-11. ©2011 AACR.

Introduction

Half of patients diagnosed with muscle-invasive urothelial bladder carcinoma develop metastases in the lungs and liver (1), resulting in poor 5-year survival rates (2). Metastatic colonization, the process of micrometastatic establishment in lymph nodes and distant organs (3), is a crucial stage of metastatic progression. Inhibiting this process could have significant impact on delaying or reducing mortality (4). A growing body of literature has provided a compelling case for CD24 as a mediator of metastatic progression (5), with metastatic progression. CD24 seems to be more than a marker, however, as we previously reported that siRNA-mediated depletion of CD24 in human tumor cell lines results in decreased viability and proliferative capacity \textit{in vitro} (6), a result supported by others (7, 8). Furthermore, use of anti-CD24 monoclonal antibody (mAb) induced CD24 protein downregulation through lysosomal degradation and reduced local tumor growth in xenograft models of colorectal cancer (8).

Despite this documented role for CD24 in primary tumors, our understanding of the function of CD24 in metastasis remains limited. Early studies have suggested that CD24 interacts with P-selectin expressed on endothelial cells and platelets (9, 10) to facilitate cellular rolling and adhesion in murine lung vasculature (11). In addition, other work has revealed that induced overexpression of CD24 can promote spontaneous metastasis in syngeneic rat models of breast cancer (12). However, several critical issues about the expression and functional role of CD24 in human metastasis remain unanswered. Here, we show for the first time in any human cancer that CD24 is increasingly expressed in metastatic tissue. We apply gene expression profiling to show that a novel xenograft model of human bladder cancer lung colonization is a related and relevant model of human disease. This model is subsequently used to evaluate the consequences that depletion and overexpression of CD24 have on lung colonization. Finally, we explore the effect of targeting CD24 with anti-CD24 mAb in preventing the outgrowth of established metastases in our human xenograft model. Together this work...
provides new knowledge on the function of CD24 in human metastasis and its suitability as a therapeutic target in this stage of the disease.

Materials and Methods

Development of the lung metastasis model and in vivo assays

UM-UC-3 human urothelial cancer cells (13) were cotransfected with pBabe (14) for puromycin selection capacity and pCMV-Lux for luciferase expression. These resultant cells were then selected with puromycin. A pooled population was subsequently termed “Luc.” Five-week-old NCr nu/nu mice (NCI-Frederick, Frederick, MD), maintained in accordance with University of Virginia ACUC guidelines, were inoculated via tail vein injection with 2.5 × 10⁶ Luc cells suspended in phenol red- and serum-free minimum essential media ([MEM) Invitrogen]. Metastatic progression was monitored via bioluminescent imaging (BLI) as described (15). Animals were sacrificed after 3 consecutive weeks of tumor visualization, whereupon lung tumors were excised and propagated in vitro with puromycin selection yielding a cell population, termed Lul-1 (luciferase lung metastasis). Lul-1 cells were injected into mice in the same manner and resultant lung metastases were explanted and propagated to create Lul-2. For experiments with established lines, s.c. tumorigenicity and experimental (tail vein) metastasis were assessed in NCr nu/nu mice as described (16, 17). Human tumor burden in the lungs, using DNA isolated from a homogenous mixture of both lungs, was assessed with human-specific quantitative PCR (qPCR) according to previously described methods (18) and human-specific 12p TaqMan probe, 5′-[(HEX)CCA][+C][GC][+A][AG][+G][CA][BHQ1]-3′, and primers: 12p F (5′-TTCACATGGCAAAATGTGTTATCC-3′), 12p R (5′-GTTGTGGGAAGGGATTAAC-3′; Sigma Genosys).

Hierarchical clustering and ranking algorithms

Duplicate, early passage isolates of RNA from UM-UC-3 Luc and LuL-2 cells were submitted for gene expression profiling using Affymetrix HG-U133A as reported (ref. 19; Affymetrix). A metastasis signature consisting of probesets exhibiting more than 3-fold and 75 unit difference in expression between the Luc and LuL-2 replicates was developed. A weighted k-nearest neighbor classification algorithm, implemented in MATLAB (code available on request; MathWorks), using the UM-UC-3 Luc cells and LuL-2 cells as training, was used to classify 91 urothelial carcinomas from a prior microarray study by Sanchez-Carbayo and colleagues (20). This classification algorithm, which used Euclidean distance as distance metric, outputted a classification call (i.e., similar to Luc or LuL-2), which was plotted for Kaplan–Meier survival analysis in Prism (GraphPad Software).

Human tumor samples and immunohistochemical analysis

Gene expression analysis data obtained from Sanchez-Carbayo and colleagues (20) contained 91 samples of bladder cancer and 48 samples of normal urothelium, of which 24 cases were of matched bladder tumor and normal urothelium. In addition, a unique set of matched primary urothelial carcinomas and nodal metastases from 70 patients was immunohistochemically (IHC) stained for CD24 with SWA11 mAb using methods described (21). Ten samples were excluded from analysis because either the primary tumor, the metastasis, or both were uninterpretable due to damaged samples. Specimens were graded for staining intensity (strength of staining) and proportion (percentage of specimen) on a scale ranging from negative to strong (0, 1+, 2+, or 3+). Total only CD24 staining was considered, as SWA11 recognizes a peptide core epitope of CD24 that invariably shows cytoplasmic staining.

Cell culture, plasmid constructs, transfection, viral transduction, and qPCR

UM-UC-3 (CRL-1749) and TCCSUP human urothelial cancer cells (19) were obtained from American Type Culture Collection (ATCC) and cultured in MEM. They were characterized by ATCC using DNA profiling [short tandem repeat (STR)], cytogenetics, and isoenzyme analysis and were used from replicate frozen stocks derived within 6 months of receipt. Human CD24 protein coding sequence cDNA was cloned into the BamHI and NotI sites of pcDNA3.1/Zero(+) (Invitrogen) vector using primers BamHI-CD24 F (5′-GGCCGATCCATGGGCAGAGC-3′) and NotI-CD24 R (5′-GAGGCCGCGCTTTAGTAGAG-3′) and stably transfected into Luc cells with FuGENE 6 (Roche). Resultant cell lines, Luc CD24− and Luc vector, were cultivated under zeocin selection for 7 days. Fluorescence-activated cell sorting (FACS) and fluorescein isothiocyanate (FITC) anti-CD24 mAb (clone ML-5; BD Biosciences) were used to isolate a population of Luc cells with high CD24 expression, termed “Luc sort hi.” Lentiviral transduction of (pLKO.1-puro) containing either CD24 short hairpin RNA (shRNA) construct or nontarget control (NT) construct (Sigma) was used in Lul-2 cells. The construct containing the CD24 targeting sequence of CCGGCCCAAGGAGTATTGCCTCGAAGACTGAAATCACGCGGTGGTGTGTTTTG was selected due to reliable and durable knockdown of mRNA and protein. A population of LuL-2 with depleted levels of CD24, LuL-2 sort lo, was isolated using a FACS method similar to that stated above. Products from reverse transcriptase PCR (RT-PCR) cDNA synthesis reactions were used in qPCR carried out in an iCycler thermocycler (Bio-Rad) with the following primers amplifying a portion of the CD24 protein coding region: CD24cds_F (5′-CTGCGGGAGATCCGACTGAAATCGCGGGGTTTTG) and CD24cds_R (5′-GTTGTGGGAAGGGATTAAC-3′). In vitro proliferation and soft agarose colony assays were carried out as described previously (22).

Multiplex imaging flow cytometry

Cells were resuspended in 50 μL of 1% bovine serum albumin (BSA) in Dulbecco’s PBS (DPBS) and 25 μL FITC anti-CD24 mAb (ML-5; BD Biosciences), which binds the protein core leucine-alanine-proline (LAP) epitope, and incubated at room temperature in the dark for 30 minutes. To ensure saturating levels of anti-CD24 mAb, 10 μL of 0.5 mg/mL
purified, unstained anti-CD24 (ML-5; BD Biosciences) was added to each suspension. Cells were pelleted and washed with 1% BSA in 1× DPBS. Next, cells were fixed in 2% paraformaldehyde for 15 minutes, washed with 1× DPBS, then permeabilized with dropwise addition of ice-cold methanol. Cells were rinsed and 50 μL of PE (phycoerythrin) anti-CD24 mAb (ML-5; BD Biosciences) was added to assess cytoplasmic CD24 staining levels. After washing, cells were passed through 80-μm nylon mesh (Small Parts Inc.) and resuspended in 75 μL of 0.1 μg/mL DAPI (4',6-diamidino-2-phenylindole; Pierce) in 1× DPBS. Analyses were conducted using an ImageStream 100 flow cytometer (Amnis) and interpreted using IDEAS 4.0 (Amnis).

Monoclonal antibody treatment

Subcutaneous tumor setting. NCr nu/nu mice were inoculated with a single injection of 5 × 10⁵ LuL-2 cells in the flank region. Mice were randomized and split into 2 cohorts. On palpation of tumor formation (7 days after inoculation), human-specific anti-CD24 mAb ALB9 (IgG1; Beckman Coulter) was delivered i.v. in a bolus dose of 5 mg/kg/d (0.1 mg in 100 μL) every 4 days, for 3 total treatments. Control animals were given 0.9% saline. This treatment course was adapted from prior trials of this same mAb in mice (23) and humans (24, 25).

Metastatic setting. Mice were inoculated via tail vein with 2.5 × 10⁶ LuL-2 cells, randomized, and split into 2 cohorts. At the time of metastatic detection by BLI, treated mice were given 5 mg/kg/d (0.1 mg in 100 μL) of anti-CD24 ALB9 and controls were given IgG1 isotype control mAb (Southern Biotech) every 4 days for a total of 4 treatments. Mice were sacrificed at 4 weeks for analysis of metastatic lung burden using human-specific qPCR or at 6 weeks for fixation in Bouin’s solution for gross metastasis analysis. For survival analysis, mice were allowed to progress until they reached Institutional Animal Care and Use Committee–accepted humane surrogates of death (cachexia >20% of control animal weight, lethargy behavioral changes, or overt respiratory/general distress).

Statistics

Cell proliferation data were compared using 2-way ANOVA. Counts of colonies growing in soft agar were compared using a 2-tailed Student t test, as were comparisons of s.c. tumor growth and metastatic tumor incidence and burden across mice cohorts. Human and murine survival analyses conducted with the Kaplan–Meier method were assessed for significance using the log-rank test. Statistical analyses were conducted in Prism 5.0 (GraphPad Software).

Results

Development and clinical validation of a bladder cancer lung metastasis experimental model

To study the functional contribution of CD24 in urothelial carcinoma lung metastasis, we developed an experimental model of this process by isolating UM-UC-3 Luc (Luc) cells that target the lungs following iterative in vivo tail vein inoculation (Supplementary Fig. S1A). Although the resultant cell population, LuL-2, exhibited slower monolayer growth than isogenic parental Luc cells (Fig. 1A), when grown in an anchorage-independent environment they produced more colonies (Fig. 1B). Mice s.c. injected with Luc cells (2 sites per mouse) developed tumors with a mean volume of 339 mm³ (median: 194 mm³), whereas mice injected with LuL-2 cells (2 sites per mouse) had a mean tumor volume of 1,649 mm³ (median: 1,443 mm³; P < 0.01; Fig. 1C). Following tail vein inoculation and weekly BLI, we detected metastases early; by 4 weeks, 7 of 8 mice injected with LuL-2 had multifocal detectable metastases compared with only 2 of 8 mice with small metastases in the Luc cohort (P < 0.01; Fig. 1D). At 6 weeks, visual examination of gross lung tumors corroborated the metastatic burden assessment obtained via BLI (Supplementary Fig. S1B). Importantly, mice inoculated with LuL-2 cells had shorter overall survival (mean of 36.9 days) compared with those injected with Luc cells (mean of 111.3 days; Fig. 1E).

To determine the ability of our system to model metastatic progression in humans, we used genome-wide expression profiling on Luc and LuL-2 cell populations to identify a metastasis signature of 59 differentially expressed probesets (Supplementary Table S1). CD24 was among the top genes upregulated in LuL-2 cells, a finding confirmed by qPCR, Western blot protein analysis, and flow cytometry (Supplementary Fig. S1C–E). Using published gene expression profiles of 91 human urothelial carcinoma cases (20), we used our 59-gene metastasis signature to classify patient outcomes based on similarity to Luc or LuL-2. Patients with classification calls similar to LuL-2 gene expression had shorter disease-specific survival compared with those patients with calls similar to Luc (P = 0.037; log-rank; Fig. 1F), thus supporting the relevance of this model in reflecting the natural progression of human bladder cancer.

Clinical evaluation of CD24 expression in human metastatic bladder cancer

Our laboratory previously identified expression of CD24 as a predictor of tumor progression in urothelial bladder cancer (6). To date, however, no analysis has been reported that directly compares the CD24 expression in primary and patient-matched metastatic tumors of any cancer type. We addressed this deficiency by evaluating IHC staining of 60 primary and metastatic tumors obtained from the same patient found at the time of radical cystectomy (Fig. 2A). CD24 expression was noted in 45 primary tumors (75.0%) and 56 metastases (93.3%; P = 0.006; Fig. 2B). We next assessed the change in CD24 expression, both in intensity and proportion, within patient-matched primary and metastatic tissues to determine whether CD24 expression increased as a function of metastatic progression. Our analysis revealed that the metastases of 37 cases (61.7%) had increased CD24 staining intensity and 23 cases (38.3%) had greater CD24 staining proportion compared with matched primary tumors (P < 0.001; Fig. 2C).

Manipulation of CD24 expression results in differential localization

Given the observed relationship between CD24 expression and human metastasis, we sought to determine whether CD24 expression is required for or simply predictive of metastasis by
developing a panel of cell lines with altered CD24 expression as follows (Supplementary Fig. S2A): First, we stably overexpressed CD24 in the Luc parental cell line by transfecting a pcDNA3.1 vector containing the cloned protein coding sequence of CD24 (Luc CD24⁺). Second, we silenced CD24 expression in metastatic LuL-2 cells using lentiviral transduction of shRNA targeted to CD24 (LuL-2 shCD24) or nontargeted scramble control (LuL-2 shNT). Third, we conducted FACS to select for tumor cell populations with high and low expression of CD24, similar to...
studies where CD24 expression is a marker for sorting tumorigenic subpopulations (26, 27). In one group, we enriched for subpopulations of Luc parental cells with high CD24 expression (Luc sort hi), whereas in another, we isolated LuL-2 cells with low CD24 expression (LuL-2 sort lo). We confirmed CD24 expression levels in the cell lines by qPCR (Supplementary Fig. S2B) and multispectral imaging flow cytometry (MIFC; Supplementary Fig. S2C and D). Surprisingly, increases in surface CD24 expression in Luc CD24⁺ and Luc sort hi cells were not coincident with increased cytoplasmic CD24 levels. Moreover, sorting for populations of cells with low surface CD24 expression (LuL-2 sort lo) did not isolate cells with as markedly reduced cytoplasmic CD24 expression as did shRNA depletion.

**CD24 expression determines acute retention of cancer cells in the lungs and lung metastasis**

Given our finding that CD24 expression is higher in patient metastases than in primary tumors, we next sought to use our cell lines with manipulated CD24 expression to evaluate the role of CD24 in metastasis. CD24 was previously reported to interact with P- and E-selectins both in culture (9, 10) and in vivo to promote adhesion of A125 adenocarcinoma cells to murine lung vasculature (11). To analyze how CD24 expression affects tumor cell retention in the lungs, we inoculated cohorts of mice (n = 16) via tail vein injection of the CD24-manipulated cell lines (Supplementary Fig. S2A). We then monitored differential cellular clearance rates from the lungs for a period from 5 to 24 hours by BLI and human-specific qPCR. Mice injected with CD24-depleted LuL-2 shCD24 cells had lower levels of total photon radiance than control LuL-2 shNT cells and had significantly reduced numbers of residual tumor cells detectable by human-specific qPCR quantification (P < 0.01; Supplementary Fig. S3A and Fig. 3A). In contrast, we did not observe a comparable effect for LuL-2 sort lo compared with LuL-2 cells, as differential clearance was only apparent at the 5-hour time point. Luc CD24⁺ cells overexpressing CD24 and Luc sort hi...
cells enriched for CD24 expression were both present in the lung at significantly higher levels than respective controls ($P < 0.01$). After 11 hours, increased CD24 levels no longer conferred lung retention benefits. Importantly, at 24 hours after inoculation, all groups had undetectable tumor presence in their lungs by qPCR, a finding that is consistent with other studies (28).

Because we have applied this assay to reliably detect 160 pg of total human DNA amid a background of 2.5 mg of murine DNA (18) and published estimates of DNA content in human cells are 1.5 to 7.0 pg of DNA (29–31), our limit of detection approaches 23 to 107 cells. Thus, CD24 seems to facilitate short-term retention of bladder cancer cells in the lungs, but this does not seem to translate into increased tumor cell presence after 24 hours.

We next examined the role of CD24 in metastatic colonization. Following tail vein inoculation, we monitored metastatic progression with BLI and used human-specific qPCR to quantify lung tumor burden at 12 (for mice injected with Luc-based cells) and 6 weeks (for those injected with more aggressive LuL-2-based cells). Overexpression of CD24 in the Luc CD24$^+$ cell line resulted in increased incidence of lung metastases (7 of 12 mice) and a greater average tumor burden by human-specific qPCR, compared with Luc vector cells (1 of 12 mice; $P = 0.043$; Fig. 3B and C). This difference in metastatic
growth was not simply a reflection of increased proliferative capacity, as after 96 hours, Luc sort hi cells proliferated at a significantly slower rate than control Luc cells \((P < 0.05)\), although Luc CD24 \(^\text{hi}\) cells also showed a trend toward slower proliferation at a similar time point \((P < 0.01; \text{Supplementary Fig. S3B})\). To evaluate the relevance of this finding in other bladder cancer cell lines, we overexpressed CD24 in the TCCSUP cell line. TCCSUP CD24 \(^\text{hi}\) cells had higher incidence and metastatic burden than control cells \((P = 0.016; \text{Supplementary Fig. S3C})\). Mice inoculated with LuL-2 shCD24 cells had reduced incidence and size of lung metastases compared with LuL-2 shNT, both by visual inspection of gross tumors (1 of 10 and 9 of 10 mice, respectively) and by human-specific qPCR analysis of metastatic burden \((P < 0.001; \text{Fig. 3B} \text{and } C)\). We observed supportive results with our FACS analysis, where Luc sort hi cells showed increased metastatic ability (6 of 10 mice) and quantitative tumor burden, compared with control Luc cells (1 of 10 mice; \(P = 0.0028\)), whereas LuL-2 sort lo cells had decreased metastatic colonization capacity compared with LuL-2 cells (6 of 10 and 10 of 10 mice, respectively; \(P = 0.0021\)). Together, these findings suggest a duality in functioning of CD24 in lung metastasis—by promoting acute retention of cancer cells in the lungs and by facilitating subsequent growth through yet unidentified signaling pathways.

**Therapeutic targeting of CD24 reduces metastasis**

The above data suggest that CD24 is both a marker of tumor aggressiveness and a promoter of metastatic tumor growth. Hence, we postulated that a mAb targeting CD24 might affect clinical outcome when used in an adjuvant or early metastatic setting. Because prior work suggested that the ALB9 mAb would be ideal for antibody-mediated therapy (32), we initially evaluated the impact of ALB9 when given to mice with s.c. tumors. Mice inoculated with \(10^6\) LuL-2 cells were treated at the first instance of tumor nodule palpation. Treatment with ALB9 resulted in lower mean tumor volume compared with control saline by 13 days after treatment initiation \((P < 0.01; \text{Fig. 4A})\). To assess the effect of ALB9 only on growth of established tumors, we examined the growth velocity in response to treatment, which revealed tumor growth rates decreased following treatment with anti-CD24 mAb and remained durably inhibited \((P < 0.01; \text{Supplementary Fig. S4A})\). These observations were consistent with differential tumor growth rates observed in nude mice inoculated with shCD24 transfected cells, which revealed that LuL-2 shCD24 cells produced significantly smaller tumors than LuL-2 shNT control cells \((P < 0.001; \text{Supplementary Fig. S4B})\).

We next evaluated the effects of ALB9 against metastatic disease by inoculating mice via tail vein with LuL-2 cells, and on detection of lung metastasis by BLI, we initiated treatment with ALB9 or control IgG1 isotype mAb. As early as 4 weeks after inoculation, a difference in lung metastatic burden across treatment groups was detectable by BLI (Fig. 4B). Anti-CD24 mAb suppressed average total photon flux, which is reflective of metastatic burden, during and immediately following treatment completion, but regrowth was observed on discontinuation of therapy (Fig. 4C). Corroborative analysis of metastatic burden by human-specific lung qPCR and visual counting at 6 weeks revealed that anti-CD24 treatment reduced tumor burden \((P = 0.0152; \text{Supplementary Fig. S4C})\) and prolonged overall survival by approximately 20\% \((P = 0.032; \text{Fig. 4D})\).

**Discussion**

Results from numerous studies suggest that CD24 is associated with poor outcomes in patients (33). Although CD24 affects in vitro anchorage-dependent and -independent (6) cancer cell growth, as well as engaging P-selectin (9) in the lungs, to our knowledge, this is the first study to show that CD24 is necessary for metastatic colonization of the lung in the context of a novel and biologically relevant human cancer xenograft model. Furthermore, our study is also the first to show that CD24 expression is higher in metastatic tumors than in their parental primaries in the same patient and, using the aforementioned human cancer xenograft model, show that CD24-directed therapy is effective in metastatic disease.

Progress in translational cancer biology relies on animal models that recapitulate the disease in patients. In most cases, such models are selected for a single phenotype, such as metastatic competence, rather than a global representation of their intrinsic biological characteristics. To address this deficiency in the field, we developed and subsequently credentialled in human cancer, a new human bladder cancer model of experimental lung metastasis. Through the use of genome-wide expression profiling, we showed that 59 gene transcripts, differentially expressed in the highly and poorly metastatic cells of this model, have excellent prediction of human cancer outcomes in 91 patients. Furthermore, the molecule we sought to study here, CD24, was among the most overexpressed transcripts in the highly metastatic cells. Using IHC to measure CD24 protein expression in patient samples, we observed high levels of CD24 in primary bladder tumors that were further increased in patient-matched nodal metastases. To our knowledge, this investigation is the first to evaluate patient-matched tissues for CD24 expression, lending further support to a prior study using unmatched prostate tissues (34) and a preliminary study in breast cancer (35). These data imply that CD24 in the primary tumor is predictive of eventual development of metastasis and that CD24 may also contribute to the metastatic process.

Use of our lung metastatic bladder cancer model allowed us to make several important observations about the role of CD24 in metastasis biology. CD24 overexpression increased and CD24 suppression significantly decreased the number of tumor cells present in the lungs of mice soon after i.v. inoculation. This result is not entirely surprising, as mucin-type glycoproteins expressed on the surface of epithelial carcinomas are widely believed to facilitate metastatic dissemination and seeding through associations with glycan family molecules (36–38). CD24, a sialoglycoprotein (5) with numerous N- and O-linked glycosylation sites (39), is a reported ligand for P-selectin (9), which seems to promote metastatic progression (40, 41). Interestingly, at 24 hours, both...
control and CD24-manipulated cells were undetectable by qPCR. Our inability to detect tumor cells by qPCR is unlikely reflective of detection thresholds, as we can reliably amplify as little as 160 pg of total human DNA (18), the equivalent of approximately 23 to 107 human cells (29–31). Thus, although CD24-mediated adhesion cannot be excluded as a contributor to clinical lung metastasis, it is unlikely to be the sole mechanism by which CD24 enhances metastasis, as CD24-dependent lung colonization is also apparently relevant.

Indeed, shRNA-mediated suppression of CD24 resulted in a profound decrease in metastatic colonization, and overexpression of CD24 successfully induced greater metastatic burden, despite its minimal impact on primary tumor growth. Together with the IHC observation that CD24 expression is nearly ubiquitous in metastases, our findings suggest that CD24 is important for metastatic outgrowth. We are now actively using this robust experimental model to further identify the mechanisms driving the colonization process.

One of the most intriguing findings in previous IHC studies of CD24 is that cytoplasmic CD24, more than membranous, is an independent prognostic marker (42–48). Indeed, high global CD24 portends a worse prognosis compared with tumors with high surface CD24 expression but lower cytoplasmic content. Our panel of cell lines and probative assays allowed us to evaluate the biological relevance of these findings. Interestingly, our MIFC analysis revealed that, despite alterations in surface expression of CD24 in Luc CD24	extsuperscript{+}, Luc sort hi, and LuL-2 sort lo cell lines, cytoplasmic CD24 levels remained unchanged. Only suppression of CD24 expression with targeted shRNA effectively reduced all cellular pools of CD24 in LuL-2 shCD24. These observations were fascinating, particularly because the most profound results were observed using the LuL-2 shCD24 cells, raising the possibility that cytoplasmic CD24 may be involved in intracellular signaling contributing to metastatic site growth. Surface CD24 may predominantly affect cellular adhesion, which is logical.

Figure 4. Treatment of s.c. or lung metastatic bladder cancer with CD24 antibody. A, i.v. treatment of bolus saline or 5 mg/kg/d (0.1 mg in 100 μL) ALB9 mAb every 4 days for 3 treatments in mice with established and palpable s.c. tumors induced by LuL-2 cells. Triangles indicate treatment delivery time points. Subcutaneous tumors were measured biweekly and volumes were calculated as defined for a spheroid (4/3πL\textsuperscript{3}W). B, ALB9 treatment of BLI detectable metastases; representative images show metastatic burden as detectable by BLI in ALB9 and isotype control (IgG1) mAb at 4 weeks. Average total photon flux is for a representative group of both ALB9 treatment (n = 8) and isotype control (n = 8) surveyed for more than 5 weeks by BLI. C, each treatment cohort included 22 mice inoculated with 2.5 × 10\textsuperscript{6} LuL-2 tumor cells via tail vein injections. Mice were treated i.v. with either isotype IgG1 mAb as a control or 5 mg/kg/d (0.1 mg in 100 μL) ALB9 mAb every 4 days for 4 treatments after detection of metastasis by BLI. A subset of these mice (n = 8) was followed weekly with BLI. **, \(P < 0.01\); ***, \(P < 0.001\). D, comparison of overall survival of mice with metastatic bladder cancer treated with ALB9 or IgG1 mAb. *, \(P = 0.032\), log-rank, 1-tailed.
because surface CD24 would be expected to participate in cell–cell interactions such as binding to P-selectin (11) on endothelial cells, thereby promoting metastatic seeding, whereas cytoplasmic CD24 may be involved in cell survival (6). Nevertheless, our results also suggest that surface CD24 does not solely contribute to early cell adhesion in the lung, as treatment with the antibody, which would only be expected to target surface CD24, was initiated days after tail vein inoculation and still had an effect on establishment of clinical metastasis. This result also shows that cytoplasmic CD24 does not entirely drive either the metastatic phenotype or growth at the s.c. site yet is a contributor in addition to that of surface CD24, as the most profound results were observed using the Lu-l.2 shCD24 cells as mentioned above, which decreases both CD24 pools. We are currently actively investigating the CD24 synthesis and processing to determine whether our cancer cells have defects on this process to account for the accumulation of CD24 in the cytoplasmic compartment.

Although several anti-CD24 mAbs exist, we used ALB9 because it targets the LAP sequence present in human, but not the murine homolog CD24 (32), which allowed for treatment of human xenografts in murine hosts. Moreover, ALB9 was evaluated in human clinical trials for the treatment of B-cell lymphoproliferative disease, where it was efficacious in promoting complete remission in a majority of patients (24, 25). Other studies have used a similar peptide core targeting anti-CD24 antibody that was delivered coincidently with s.c. implantation of HT-29 colorectal carcinoma cells, which reduced subsequent tumor growth in nude mice (8). Our study is the first to evaluate anti-CD24 mAb as a treatment for established tumors and as an antimetastatic therapy. Importantly, ALB9 was efficacious in treating established metastases, despite attaching to CD24 in a region distinct from the binding site of P-selectin (9), which strongly supports that CD24 is functioning to retard metastatic colonization and not merely CD24-mediated endothelial adhesion. This observation sets the stage for use of such antibodies in the adjuvant or early metastatic settings for high-risk bladder cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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