A Bispecific Antibody Targeting the $\alpha_\text{v}$ and $\alpha_5\beta_1$ Integrins Induces Integrin Degradation in Prostate Cancer Cells and Is Superior to Monospecific Antibodies

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ABSTRACT

Fibronectin-binding integrins $\alpha_5\beta_1$ and $\alpha_v$ collaborate in prostate cancer–bone stromal interactions relevant to the colonization of the bone marrow microenvironment. Combinatorial inactivation of these integrins on prostate cancer cells was assessed. Monospecific antibodies to $\alpha_5\beta_1$ and $\alpha_v$ integrins alone (MAb) and in combination (cMAb), and a bispecific antibody that simultaneously targets $\alpha_5\beta_1$ and $\alpha_v$ integrins (BsAb$\alpha_5\beta_1$/av) were compared in assays of chemotaxis, clonogenic survival, and induction of endothelial migration. Cellular expression of integrins, their transcription, translation, and degradation fate was compared. The BsAb$\alpha_5\beta_1$/av was superior to MAb and cMAbs in abrogating adhesion, migration, clonogenic survival, and induction of endothelial migration responses by prostate cancer cells. Integrin upregulation observed with MAb or cMAbs was abrogated with the BsAb$\alpha_5\beta_1$/av. Loss of integrin expression was uniquely induced by the BsAb$\alpha_5\beta_1$/av and blocked by lysosomal inhibition.

Implications: A novel and effective combinatorial strategy to target $\alpha_5\beta_1$ and $\alpha_v$ integrins is defined for translational studies.

Visual Overview: http://mcr.aacrjournals.org/content/molcanres/18/1/27/F1.large.jpg.

Introduction

The preferential colonization of the bone marrow microenvironment by disseminated prostate cancer cells underpins its lethal metastatic phenotype. Deconvolution of the molecular mechanisms that mediate this behavior can define novel therapeutic strategies to improve mortality and morbidity from the disease. Bone marrow– derived mesenchymal stromal cells have been implicated as architects of both the hematopoietic (1) and bone metastatic niche (2). Earlier studies indicated that human bone marrow–derived mesenchymal stromal cells (hBM-MSC) induce a strong chemotactic and adhesive response in prostate cancer cells. The relevant bioactive fraction of the hBM-MSC secretome was purified and proteolytic fragments of fibronectin (FNFr) signaling via the $\alpha_5\beta_1$ integrin in prostate cancer cells identified as the basis of the chemotactic response (3). Genetic inactivation of the $\alpha_5$ integrin induced programmed cell death in prostate cancer cells indicating a role in adhesion-mediated survival (4). Accordingly, the FNFr–$\alpha_5\beta_1$ integrin interaction was proposed as a seed-and-soil mechanism of bone colonization by prostate cancer (3).

We hypothesized that the $\alpha_v$ integrin, an alternative fibronectin-binding integrin (5) cooperates with the $\alpha_5\beta_1$ integrin in mediating the metastatic niche interactions of prostate cancer cells. By comparing the impact of monospecific neutralization of $\alpha_5\beta_1$ and $\alpha_v$ integrins alone (MAb) and in combination (cMAb) to a first-in-class integrin-targeting bispecific antibody, BsAb$\alpha_5\beta_1$/av,
we find that the BsAb5β1/αv optimally neutralizes tumor–stromal interactions with a novel mechanism of action.

Materials and Methods
Prostate cancer, stromal and endothelial cell lines, and culture
Prostate cancer cell lines PC-3, DU-145, LNCaP, VCaP, and C4-2B and human umbilical vein endothelial cells (HUVEC) were obtained from ATCC and the Characterized Cell Line Core Facility (MD Anderson Cancer Center, Huston, TX). Plastic adherent hBM-MSCs were generated from human bone marrow aspirates (Lonza) as described previously. Cell lines represent genomically and phenotypically diverse prostate cancer cell lines: integrin α5/αv coexpressing lines include bone-derived PTEN-null androgen receptor (AR)-negative PC-3 cells, bone-derived PTEN-null AR-positive hormone-resistant C4-2B cells, lymph node-derived PTEN-null AR-positive hormone-sensitive LnCaP cells, and PTEN wild-type AR-negative DU-145 cells. TMPRSS2-ERG-positive, PTEN wild-type AR-positive VCAP cells lack membrane α5 expression.

Generation and validation of a bispecific antibody to integrins α5β1 and αv
A genetic construct was designed to express a bispecific antibody that targets αv (6) and α5β1 integrins (7). The construct was expressed in 293T cells, and the resulting supernatant was purified by protein A chromatography (Creative Biolabs). A similar approach was used to generate α5β1 and αv IgG control antibodies with the same antigen-binding sequences as BsAb5β1/αv. Further description of structure, purity, and binding of BsAb5β1/αv is provided in Supplementary Fig. S1.

Generation of conditioned media
hBM-MSC conditioned media (CM) was generated as described previously (3). Briefly, confluent hBM-MSCs were cultured in serum-free media with CM harvested after 24 hours and gently centrifuged before being stored at 4°C.

Cell migration and adhesion assays
Prostate cancer adhesion and migration assays were performed as described previously (3). Briefly, migration of cancer cells to hBM-MSC CM was identified previously (3). Furthermore, coculture of prostate cancer cells with hBM-MSCs strongly induces the migration of human endothelial cells. Using these in vitro tumor–niche interaction models, we tested the hypothesis that combinatorial blockade of integrins αv and α5 would be superior to single integrin blockade alone across a panel of genomically diverse, integrin α5/αv coexpressing prostate cancer cells. As hypothesized, combined α5 and αv blockade with dual MAbs was superior to individual single agents in inhibiting prostate cancer cell migration, adhesion, and induced endothelial migration in each of these cell lines (Fig. 1A).

Results
Combinatorial integrin α5 and αv blockade is superior to blockade of either integrin in inhibiting prostate cancer cell chemotaxis and induced endothelial migration
A strong chemotactic and adhesive response of prostate cancer cells to hBM-MSC CM was identified previously (3). Furthermore, coculture of prostate cancer cells with hBM-MSCs strongly induces the migration of human endothelial cells. Using these in vitro tumor–niche interaction models, we tested the hypothesis that combinatorial blockade of integrins α5 and αv would be superior to individual single agents in inhibiting prostate cancer cell migration, adhesion, and induced endothelial migration in each of these cell lines (Fig. 1A).

Generation of a bispecific antibody to integrins α5β1 and αv
On the basis of the hypothesis that a bispecific antibody targeting these two integrins would be superior to a combination of MAbs, we designed and generated a bispecific antibody. The molecule is composed of an IgG that targets the αv integrin (6) and a single-chain variable fragment (scFv) that targets the α5β1 integrin (ref. 7; Supplementary Fig. S1A). By including a pan-αv integrin–targeting sequence, the problem of diverse αv heterodimers with overlapping functions was skirted whereas α5β1 is the obligate heterodimer of α5 integrin. BsAb5β1/αv purity was demonstrated by gel electrophoresis (Supplementary Fig. S1B). BsAb5β1/αv binding to target integrin receptors was correlated with commercially available αv and α5 antibodies across a panel of variably expressing α5 and αv cell lines (Supplementary Fig. S1C). High affinity, specific binding of BsAb5β1/αv to target integrins was demonstrated using the Biacore platform (Supplementary Fig. S2).

BsAb5β1/αv is superior to monospecific combination in inhibiting prostate cancer cell chemotaxis and induced endothelial migration
We hypothesized that BsAb5β1/αv would be superior to cMABs in assays of tumor–hBM-MSC interaction. Bs5/αvAb was superior to the combination of sequence-matched monospecific IgG controls...
Figure 1.
Combinatorial integrin α5 and αv blockade is superior to monospecific blockade and BsAbα5β1/αv is superior to combinatorial integrin blockade in inhibiting prostate cancer cell migration, adhesion, and induction of endothelial migration. PC-3, DU-145, or C4-2B cells were harvested and treated with 50 μg/mL of MAbs or cMAbs (A) or 10 μg/mL of BsAbα5β1/αv or cMAbs (20μg/mL total; B) for 20 minutes on ice before use in functional assays. *, P < 0.05; **, P < 0.01; †, P < 0.001; ††, P < 0.0001.

(MAbs or MAbs) in inhibiting prostate cancer cell migration, adhesion, and induced endothelial migration (Fig. 1B). A scFv control was not considered to be an optimal control as its half-life in vivo is expected to be low given its low molecular weight and susceptibility to rapid renal clearance.

BsAbα5β1/αv treatment uniquely induces loss of integrin expression and/or blocks integrin upregulation in prostate cancer cells

In assessment of the pharmacodynamic effects of the BsAbα5β1/αv, a marked loss of total cellular integrin αv and α5 in PC-3 cells at 48 hours following treatment was observed (Fig. 2A). This reduction in expression was not observed with MAbs or cMAbs. In DU-145, C4-2B, and VCaP cells, treatment with MAbs and/or cMAbs, variably resulted in upregulation of either or both integrins. However, in each case this upregulation was either abrogated or markedly inhibited by BsAbα5β1/αv treatment (Fig. 2A). In contrast, preferential impact of the BsAbα5β1/αv on focal adhesion kinase, Akt, or Erk signaling was inconsistent across cell lines (Supplementary Fig. S3). MMP-14, a master regulator of matrix degradation, MMP-2 and TGF-beta activation, was strongly downregulated with cMAbs and the BsAbα5β1/αv but not MAbs (Supplementary Fig. S4).

Regulation of integrin expression dynamics by BsAbα5β1/αv is post-translational and related to altered trafficking and lysosomal degradation of target integrins

Using PC-3 prostate cancer cells as a model line, we investigated the mechanism by which the BsAbα5β1/αv induced a marked decrease in cellular integrin expression. We initially hypothesized that these dynamic changes might be explained by rapid endocytosis and degradation of antibody-bound receptor as seen previously with other antibodies. However, BsAbα5β1/αv treatment resulted in slow progressive loss of αv membrane expression observed over 24–48 hours post-treatment (Fig. 3A). This loss of membrane expression correlated with diminished total cellular expression of αv (data not shown).

To assess the fate of these integrins more closely, we assessed whether integrin synthesis or degradation were specifically impacted by BsAbα5β1/αv treatment. Quantitative reverse-transcription PCR studies showed no significant change in ITGAV or ITGAV transcript expression following either MAbs, cMAbs, or BsAbα5β1/αv treatment (Fig. 3B). Treatment with cycloheximide, an inhibitor...
of protein translation, failed to abrogate the differential impact of BsAb5β1/αv on target expression (Fig. 3C), indicating the regulation was post-translational. Because the major post-translational regulator of integrin expression is transition from recycling pathways to lysosomal/endosomal degradation (8), we hypothesized that BsAb5β1/αv induced preferential lysosomal degradation of bound target receptors. Treatment with chloroquine, a disruptor of lysosomal integrity and function, blocked the downregulation of integrin expression observed with BsAb5β1/αv therapy (Fig. 3D; Supplementary Fig. S6).

**Discussion**

Our studies have implicated fibronectin-binding α5 and αv integrins in the key interactions of prostate cancer cells with hBM-MSCs, the putative metastatic niche-regulating cell in the bone microenvironment. We show that combined antibody blockade of these integrins is superior to single-integrin blockade and that a bispecific antibody strategy optimally abrogates chemotaxis, clonogenic tumor survival, and membrane depletion, blocked upregulation, and induced lysosomal degradation of target integrins by the BsAb5β1/αv contrast strongly with upregulated integrin expression following single or combined monospecific antibody therapy. The data presented in this article provide foundational data to advance in vivo and translational studies of the novel BsAb5β1/αv strategy.

From a historical perspective in targeting integrins in prostate cancer, the αvβ1 mAb volociximab was abandoned in the absence of discernible clinical efficacy in a limited set of solid tumor studies (9). Only a handful of patients with prostate cancer were included, and the efficacy data are insufficient to draw conclusions. Synthetic peptide approaches to block the αvβ1 integrin (10) did not advance in clinical trials and it is unclear if peptide-based competitive binding strategies are capable of interdicting both ligand-dependent and ligand-independent integrin functions, both of which are implicated in tumor survival and dissemination (11). From other lines of investigation, the αv integrin has been shown to be expressed in primary tumors, mediate bone homing in experimental models, mediate interaction with endothelial cells and bone matrix components to drive tumor progression, and endow prostate cancer cells with stem-repopulating capacity (12). Integrin αv was targeted with the cyclic peptide ciliengitide but without significant clinical impact (13). Contrasted to αvβ1, the αv integrin has multiple heterodimeric forms implicated in the...
**Bispecific Antibody Targeting of α5β1 and α5β1 Integrins**

**Figure 3.**
Regulation of integrin expression dynamics by BsAb5β1/αv is post-translational and related to altered trafficking and lysosomal degradation of target integrins. **A,** In PC-3 cells, BsAb5β1/αv treatment results in progressive loss (over 24 hours+) of membrane αv expression. **B,** ITGA5 and ITGAV transcript expression is not significantly altered (fold change >1.5 or <0.5) in PC-5 cells following MAb, cMAb, or BsAb5β1/αv treatment. Blockade of protein translation with cycloheximide failed to abrogate the differential impact of MsAbs or BsAbs/αvβ1 on integrin expression in PC-3 cells (C). **D,** Lysosomal inhibition with chloroquine blocked the downregulation of integrin expression with BsAb5β1/αv therapy in PC-3 cells. 10 μg/mL cycloheximide; 50 μmol/L chloroquine.

Progression of prostate cancer including αvβ1 and αvβ6 (14, 15). The restricted αvβ3 and αvβ5 heterodimeric targeting by celeritide, its short half-life administered by intermittent intravenous infusion, and rapid adaptive resistance by membrane recycling of αvβ1 (16) likely contribute to treatment failure. A pan-αv mAb, abituzumab, demonstrated intriguing biological activity by delaying progression of bone metastases in men with metastatic castration-resistant disease. However, overall survival differences were not demonstrable (17). These data suggest that the αv integrin is probably implicated in the biology of prostate cancer progression in bone but that adaptive resistance to monotherapy likely dictates the lack of survival advantage. Rapid membrane recycling of αvβ1 in response to αv blockade with celeritide offers evidence of cross-regulation of these two integrins to account for drug resistance. The enriched expression of αv and αv integrins in disseminated tumor cells recovered from bone marrow aspirates and in pathologic specimens of bone metastases in prostate cancer (18, 19) lends additional rationale to the importance and necessity of targeting both integrins.

The potential advantages of a bispecific antibody targeting α5β1 and αv over a combination of MABs include harmonized pharmacokinetic and pharmacodynamic considerations within one molecule. Lack of steric hindrance between two large molecules targeting proximate targets in the cell membrane, cross-linking of targets leading to increased binding affinity and superior on-target time, and the induction of novel biology such as induced degradation of target proteins. More than one of these mechanisms of action likely account for the markedly improved *in vitro* activity of the BsAb5β1/αv over MABs.

In addition, upregulated cellular integrin α5 and/or αv expression noted with either MABs or cMAbs may represent an autoregulatory cellular adaptive response that could generate resistance to therapy. In VCAP cells, which are α5 membrane-negative, cellular integrin α5 expression is robustly upregulated by αv blockade demonstrating that upregulation of integrins emerges despite lack of detectable baseline expression. We have not noted an increase in membrane-specific expression of integrins that correlates with this cellular upregulation, suggesting that intracellular pooling of these integrins in endosomes from impaired recycling may occur primarily as a result of MAB therapy. Persistent low-level recycling or intracellular signaling from endosomal integrins (20) may account for resistance to MAB or cMAb therapy. Such resistance pathways are probably mitigated by the BsAb5β1/αv, which triggers lysosomal degradation, loss of intracellular integrins, and membrane recycling.

The precise mechanism by which BsAb5β1/αv induces degradation is unknown, but we speculate that the steric consequences of BsAb5β1/αv binding may destabilize the Rab family-regulated machinery required for integrin recycling (8). Exceptionally, combined MABs may also trigger lysosomal degradation of integrins, as demonstrated in C4-2B cells. The BsAb5β1/αv effect on target integrin expression is slower to develop, that is, over 48 hours but also long-lasting, reversing after 7 days. These remarkably slow pharmacodynamic changes after a single brief exposure of tumor cells to the BsAb5β1/αv have challenged the accurate tracking of integrin fates with traditional immunofluorescent tagging. When our data are taken together, the BsAb5β1/αv may be seen to impose an early effect on chemotaxis, clonogenic survival, and induction of endothelial
migration secondary to highly effective dual blockade of the membrane integrins on tumor cells followed by a persistent late effect driven by degradation and loss of the tumor integrins.

Integrin depletion offers a method for pharmacodynamic monitoring of tumor cells in vivo. There is lack of consistency in the impact of BsAb\(\alpha v\beta 1/\alpha v\beta 5\) on downstream signaling intermediates which may reflect FAK-dependent and FAK-independent integrin signaling (11) and the varied genetic backgrounds of these prostate cancer cells. Notwithstanding these signaling and pharmacodynamic variations, the functional consequences of the BsAb\(\alpha v\beta 1/\alpha v\beta 5\) have been consistent and justify further translational study of this first-in-class strategy to target integrins in prostate cancer. The bispecific antibody strategy may also prove relevant as a molecular therapeutics strategy to target other components of the enabling tumor microenvironment regulated by \(\alpha v\) and \(\alpha v\beta 1\) integrins including immunosuppressive cancer-associated fibroblasts, bone remodeling cells, and blood vessels. Other neoplastic and nonneoplastic disease states including pathologic fibrosis and angiogenesis in which these two integrins have been implicated may also be susceptible.

References


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