Introduction

Although various intrinsic mechanisms, such as genetic alterations, histone deacetylase-mediated chromatin modifications, activation of multidrug resistance transporters (1–3) and enrichment of cancer stem/initiating cells (4), may determine responses to anticancer therapeutics, accumulating evidence has clarified the role of tumor microenvironments in the regulation of therapeutic outcomes (5, 6). Conversely, tumor cells may modify the biologic properties of stromal cells, endothelial cells, and host immunity in local microenvironments, resulting in further tumor progression and a worse prognosis (7, 8). Therefore, the acquisition of anticancer drug resistance may render tumor cells with the ability to modulate their microenvironments in a paracrine fashion, further enhancing survival signals and the progression of tumors.

Integrin-αvβ3 is expressed on tumor cells, endothelial cells and stromal cells, implying broad activities on tumor microenvironments (9). Integrin-αvβ3 plays a critical role in triggering invasive and metastatic activities through the coordinated activation of multiple oncogenic signals and thus contributes to the resistance to specific molecular target therapies (9–11). Integrin-αvβ3 is also critically involved in angiogenesis in cooperation with several growth factors such as VEGF (12). In addition, integrin-αvβ3 on myeloid cells regulates tissue inflammation and autoimmunity by regulating helper T cell differentiation and cytokine profiles (13–15). Due to its critical involvement in inflammation and cancer, specific inhibitors targeting integrin-αvβ3 have been developed for treatment of cancer at advanced stage, and they have shown significant clinical effects (16, 17). Therefore, there is a critical need to address the optimal conditions and therapeutic options in which integrin-αvβ3 inhibitors should be used to treat malignant disorders.

Here, we present the first evidence that DNA damage signals are responsible for triggering integrin-αvβ3 upregulation on drug-resistant tumor cells. As machineries by which integrin-αvβ3 promotes tumorigenicity, the resistant tumor cells triggers the impairment of DC immunogenicity and antitumor immunity due to the integrin-αvβ3-mediated recognition of live tumor cells. Our findings reveal a unique role for integrin-αvβ3 in linking drug resistance with immune evasion and implicate integrin-αvβ3 as a novel therapeutic target in cancer patients.

Abstract

Although the tumor microenvironment plays a critical role in tumor progression and metastasis, the relationship between chemotherapy resistance and modulation of the tumor microenvironment remains unclear. Here, we report a novel mechanism showing how constitutive DNA damage signals in therapy-resistant tumor cells suppress antitumor immunity in an integrin-αvβ3-dependent manner. Integrin-αvβ3 was upregulated on various therapy-resistant tumor cells through chronic activation of ATM/Chk2- and NFκB-mediated pathways. Inhibiting tumor-specific expression of integrin-αvβ3 improved therapeutic responses to anticancer drugs by stimulating endogenous host immune systems. Mechanistic investigations revealed that tumor-specific integrin-αvβ3 expression targeted dendritic cells, facilitating their ability to phagocytose viable therapy-resistant tumor cells and thereby impaired their ability to cross-prime antigen-specific T lymphocytes. Together, our results clarify the detrimental effects of constitutive DNA damage signals to chemosensitivity and antitumor immunity. Furthermore, these findings suggest that integrin-αvβ3 targeting may benefit patients’ refractory to current anticancer regimens by defeating DNA damage signaling-induced immune escape. Cancer Res; 72(1); 56–63. ©2011 AACR.

Authors’ Affiliations: 1Research Center for Infection-Associated Cancer; Divisions of 2Molecular Immunology and 3Signaling in Cancer and Immunology, Institute for Genetic Medicine, Hokkaido University; 4Department of Medical Oncology, Hokkaido University Graduate School of Medicine, Sapporo; and 5Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Masahisa Jinushi, Institute for Genetic Medicine, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060-0815, Japan. Phone: 81-11-706-6073, Fax: 81-11-706-6071; E-mail: Jinushi@igm.hokudai.ac.jp

doi: 10.1158/0008-5472.CAN-11-2028

©2011 American Association for Cancer Research.

ATM-Mediated DNA Damage Signals Mediate Immune Escape through Integrin-αvβ3-Dependent Mechanisms

Masahisa Jinushi1, Shigeki Chiba1,2, Muhammad Baghdadi1,3,4, Ichiro Kinoshita4, Hirotoshi Dosaka-Akita4, Koyu Ito5, Hironori Yoshiyama1, Hideo Yagita5, Toshimitsu Uede2, and Akinori Takaoka1,3

Microenvironment and Immunology

Cancer Research
Materials and Methods

Mice and tumor cells

ATM-deficient mice were backcrossed at least 9 generations onto the C57BL/6 strain and housed under specific pathogen-free conditions. Genotypes were confirmed by PCR, and the experiments were conducted as described previously (18, 19). C57BL/6 and nonobese diabetic/severe combined immunodeficient (NOD-SCID) animals were purchased from SCL and Charles River, respectively. All experiments were conducted under a protocol approved by the animal care committees of Hokkaido University.

The tumor cells (MC38, HCT116 for colorectal carcinoma cells; MCF-7 for breast carcinoma cells; A375 and B16 for melanoma cells; NCI-H1975, PC3 for NSCLC cells; Hep3B for hepatoma cells) were obtained from the American Tissue Culture Collection (ATCC). K029 melanoma cells were established from the metastatic lesion of advanced patients as previously described (20). The therapy-resistant variants were established by the exposure of increasing concentrations of therapeutic agents (50–100 times higher than maximal toxic concentrations). The cell lines described above were authenticated by the Central Institute for Experimental Animals (Kawasaki, Japan) for interspecies and mycoplasma contamination by PCR.

Human samples

The clinical protocols for this study project were approved by the committees in Institutional Review Board of Hokkaido University Hospital (Approval number: 10-0114). Pleural effusion samples were obtained from patients with stage IV non–small cell lung carcinomas (NSCLC) after written informed consents had been obtained. The cells were isolated by Ficoll–Hypaque density centrifugation, and further purified as EpCAM+ epithelial tumor cells from pleural effusion.

Evaluation of integrin-αvβ3 expression

The cell lines and primary tumor infiltrates obtained from patients with advanced cancer were analyzed by flow cytometry using mAbs against human integrin-αvβ3 (clone: LM690; Millipore). In primary cells, the epithelial tumor cells were fractionated as EpCAM-positive populations. All clinical protocols received approval from Institutional Review Board of Hokkaido University Hospital (Approval number: 10–0114).

Immunoblotting

A375 melanoma cells or those resistant to PLX-4270 were stimulated with 50 nmol/L PLX-4270, and the cell lysates were then subjected to Western blotting with Abs specific for antiphospho-chk2 (Thr165), phospho-ATM (Ser138; Cell Signaling Technologies) and integrin-αvβ3 (Millipore). β-Actin was used as a loading control to check the integrity of each sample.

siRNA transduction

The design, preparation, and transduction of siRNA vectors were carried out as described previously (10). In brief, expression plasmids containing human chk-2 siRNA, integrin-β3 and ATM siRNA was obtained from Thermo-Scientific, and transfected into tumor cells according to the manufacturer’s instructions. The gene knockdown efficacy was assessed by protein immunoblot analysis, and proved to be more than 95%.

In vivo antitumor activities of BRAF inhibitor and anti-integrin-αv mAb

For in vivo tumor experiments, C57BL/6 or NOD-SCID mice were challenged subcutaneously in the flank with B16 melanoma cells transfected with V600E BRAF (B16.V600E) or those resistant to PLX-4270 (1 × 105) on day 0. For the therapy model, mice were injected with 250 μg of anti-mouse integrin-αv mAb (RMV-7) twice a week and 250 mg/kg of PLX-4270 (Merck-Calbiochem) 3 times a week. Tumor growth was measured every 5 days. In some instances, intratumoral administration of bone marrow–derived dendritic cells (BMDC; 1 × 106/mouse) was carried out with PLX-4270 and anti–integrin-αv mAb to examine the effect on antitumor responses.

Immune phenotypic assay

The frequency and phenotype of T lymphocytes in tumor draining lymph nodes (TDL) were analyzed by flow cytometry using anti-CD4, anti-CD8, anti-CD44, and anti-CD62L Ab (BD-Bioscience). For intracellular staining, TDLs were dispersed and pretreated with anti-CD3 and anti-CD28 agonistic Ab (BD-Bioscience) for 24 hours. The cells were then treated with brefeldin-A (Sigma-Aldrich), stained with anti-CD4 or anti-CD8, fixed, permeabilized with Cytofix/Cytoperm buffer, and stained again with PE-conjugated Abs for Foxp3, IFNγ, IL-10, and IL-12 were quantified by ELISA (BD Bioscience) using supernatant obtained from cultured TDL or BMDCs. The frequency of each immune cell population was determined by flow cytometry.

Phagocytosis assays

B16 melanoma cells or those resistant to PLX-4270 or dacarbazine were untreated or exposed to γ-irradiation (50 Gy) to trigger apoptosis. The live or apoptotic cells were labeled with PKH26 red fluorescence dye (Sigma-Aldrich), as described above. BMDCs were generated from bone marrow cells by culturing for 7 days in the presence of conditioned media (days 0, 2, 4, and 6) from CHO cells secreting GM-CSF. BMDCs were cocultured with the labeled live or apoptotic cells for 4 hours and evaluated for phagocytosis efficiency by flow cytometry. In some experiments, the tumor cells were pretreated with anti–integrin-αv mAb (30 μg/mL) for 30 minutes before the coculture to evaluate the contribution of integrin-αvβ3 to tumor cell engulfment.

Cross-priming assays

BMDCs were cocultured with untreated or irradiated B16-OVA cells (1:10 ratio) that were labeled with PKH26 in 12-well round-bottom plates for 4 hours and phagocytosis was determined with flow cytometry. CD11c+ cells were isolated by magnetic cell sorting (Miltenyi Biotec), and cocultured with naïve CD8+ T cells from the spleens of OT-1 mice. Intracellular IFNγ expression in T cells was then determined by flow cytometry. In some experiments, the tumor cells were...
pretreated with anti-integrin-αv mAb (30 μg/mL) for 30 minutes before the coculture to evaluate the contribution of integrin-αvβ3 to the cross-priming of OVA-specific CTLs.

Statistics
The differences between 2 groups were determined with the Student \( t \) test or the 2-sample \( t \) test with Welch’s correction. The differences among 3 or more groups were determined with a 1-way ANOVA. The \( P \) values less than 0.05 are considered as statistically significant. \(*\) \( P < 0.05\), ns: not significant.

Results

Expression of integrin-αvβ3 on chemoresistant tumor cell variants
Although it has been established that integrin-αvβ3 plays a critical role in tumorigenesis through multiple oncopgenic signaling pathways (9), it remains unknown whether it affects tumorigenic activities during the course of anticancer therapies. Thus, we generated stable drug-resistant variants of tumor cells of various origins through prolonged exposure to increasing concentrations of anticancer drugs and examined the expression levels of integrin-αvβ3 on the tumor cells. The drug-resistant phenotypes were confirmed with decreased caspase-3 activities in resistant variant of tumor cells (Supplementary Fig. S1). Integrin-αvβ3 was expressed at much higher levels on various tumor cells with resistance to cytotoxic and molecular targeting agents than on parental cell lines. This is represented by K029 melanoma cells resistant to BRAF kinase inhibitor PLX-4720 and HCT116 colon carcinoma cells resistant to epithelial growth factor receptor (EGFR) mAb (Cetuximab; Fig. 1A and B). Furthermore, we found that integrin-αvβ3 expression was highly expressed on tumor cells obtained from patients with NSCLC who received multiple rounds of chemotherapy but did not respond clinically (TX) compared with those before initial chemotherapy (Fig. 1C). Thus, integrin-αvβ3 induction in tumor cells seems to be correlated with resistance to anticancer drugs.

DNA damage signals are critical for integrin-αvβ3 induction in therapy-resistant tumors
To further examine the functional significance of integrin-αvβ3 on tumor chemoresistance, we focused on melanoma cells resistant to anticancer drugs because integrin-αvβ3 has been associated with progression in patients with melanoma (21, 22). In addition, BRAF kinase inhibitors have emerged as a new option against advanced melanomas in the clinic (23), but therapeutic responses were mostly transient and closely associated with the emergence of drug resistance (24, 25). Although recent evidence has unveiled the intrinsic alterations of oncogenic signals in BRAF inhibitor-resistant melanomas, BRAF inhibition also manipulates host immune responses, underscoring their role in the regulation of tumor microenvironments (26). Thus, it is critical to address the additional mechanisms and its biological consequences in which melanoma cells acquire resistance to therapeutic regimens including BRAF inhibitors.

Various cellular stresses, including UV, \( γ \)-irradiation, and cytotoxic drugs, trigger DNA damage responses through coordinated interplay of the ATM-chk2 and ATR-chk1 pathways, which may regulate the therapeutic antitumor responses induced by chemotherapy and radiotherapy (27). To our surprise, the ATM-chk2 activation triggered by BRAF inhibition was significantly stronger in resistant A375 cells (A375-BRAFIR) than sensitive A375 tumor cells. Furthermore, A375-BRAFIR cells but not their sensitive counterparts expressed the active form of ATM/chk2 under steady state (Fig. 2A). The ATM activities in sensitive A375 cells were maximized 12 hours after PLX-4720 stimulation or increased in a dose-dependent manner, but activation levels remained constant in A375-BRAFIR cells at all concentrations and time course of the treatment (Supplementary Fig. 2B). Several siRNA-mediated targeting of ATM genes suppressed integrin-αvβ3 expression in various therapy-resistant but not naive melanoma cells (A375, K008, B16) and colon cancer cells (MC38; Fig. 2B and Supplementary Fig. S3A), suggesting that constitutive activation of ATM-chk2-mediated DNA damage pathways is required for integrin-αvβ3 upregulation.

The mRNA levels of integrin-αv and β3 were higher in A375-BRAFIR than A375 at steady state, and treatment with PLX-4720 increased the expression levels in sensitive A375 cells although less than A375-BRAFIR cells. Furthermore, treatment with ATM inhibitor significantly depressed integrin-αv and β3 expression in B16-BRAFIR (Supplementary Fig. S4). These results indicated that ATM regulates integrin-αvβ3 expression at transcriptional levels. In stark contrast, the knockdown of ATR or p53 did not repress integrin-αvβ3 expression (Fig. 2C). Furthermore, integrin-αvβ3 expression in A375-BRAFIR cells was most depressed with NF-κB inhibitor BAY11-7082 at similar extent to ATM inhibitor KU55933, consistent with previous findings that genotoxic stress-mediated activation of ATM was linked with the NF-κB signaling cascade, which serves as a transcriptional activator of integrin-αv (Ref. 28; Fig. 2D).

To further define the role of DNA damage pathways in modulating integrin-αvβ3 expression, peripheral blood leukocytes from ATM-knockout or wild-type mice were exposed to \( γ \)-irradiation, and integrin-αv expression was evaluated on apoptotic (Annexin-V+) and surviving (Annexin-V−) populations, in which the proportion of apoptotic cells was similar in ATM-deficient and wild-type cells (Fig. 2E). Integrin-αv expression was upregulated on surviving leukocytes from wild-type but not ATM-deficient mice, but this induction was abrogated in apoptotic leukocytes from both wild-type and ATM-knockout mice (Fig. 2E). These results indicate that the ATM-dependent DNA damage pathway was necessary for triggering integrin-αvβ3 without oncogenic activation or transformation.

We next evaluated the involvement of DNA damage signals in the \( in \) \( vivo \) antitumor activities of BRAF kinase inhibitor. For this purposes, we utilized B16 melanoma stably transfected with mutant V600E BRAF (B16-V600E) because murine melanoma cells including B16 rarely bear V600E BRAF activating mutations. We confirmed that B16-V600E conferred sensitivities to BRAF kinase inhibitors (Supplementary Fig. S1).
B16-V600E cells were further stimulated with PLX-4270 to create their drug-resistant variant (B16-BRAFIR). The introduction of inrtegrin-αvβ3 siRNA, which is efficiently reduced integrin-αvβ3 expression on cell surface (Supplementary Fig. S3B), triggered apoptotic cell death in B16-BRAFIR cells but less than in B16-V600E treated with PLX-4720 alone. The specific ATM inhibitor (KU53955) also decreased apoptosis in B16-BRAFIR treated by PLX-4720 with or without integrin-αvβ3 siRNA (Supplementary Fig. S5). These results showed that inhibition of tumor-derived integrin-αvβ3 expression conferred anticanic agents with the ability to kill therapy-resis-
tant tumors via ATM-dependent mechanisms.

Furthermore, PLX-4720 exhibited little antitumor effect on B16-BRAFIR in vivo, but concurrent administration of neutralizing anti-integrin-αv mAb with PLX-4720 significantly reduced the tumor burden in C57BL/6 mice (Fig. 2F). However, PLX-4720 was sufficient to suppress ATM-deficient B16-BRAFIR tumor growth without integrin-αv mAb treatment. In either case, integrin-αv mAb alone was insufficient to trigger antitumor activities against B16-BRAFIR (Fig. 2F). The in vivo resistance to anticanic agent and its reversal by anti-integrin-
αv mAb or ATM inhibition was also confirmed in MC38 colon cancer cells resistant to the chemotherapeutic drug CPT-11 (MC38-CPT11R) (Fig. 2G), in which integrin-αvβ3 was upregulated in an ATM-dependent manner (Fig. 2B). Together, these results show that integrin-αvβ3 is important for triggering anticancer drug resistance in tumor cells via the ATM-
chk2-mediated pathway.

**Therapy-resistant tumors suppress host antitumor immunity in an integrin-αvβ3–dependent manner**

The activation of DNA damage signals causes proinflammatory cytokine secretion from senescent tumor cells (29, 30) and activates innate immune signals through induction of the stress-related molecules such as NKG2D ligands (31). Therefore, we evaluated the role of integrin-αvβ3 triggered by DNA

---

*Figure 1.* High expression of integrin-αvβ3 in tumor cells that acquired resistance to anticancer drugs. A, the cell surface expression of integrin-αvβ3 (green line) was examined in K029 melanoma cells with acquired BRAF-kinase inhibitor resistance (K029-BRAFIR), and HCT116 colon carcinoma cells and HCT116 with acquired anti-EGFR mAb resistance (HCT116-EGFRIR). The staining with isotype-matched Ig was overlaid as filled histograms. B, the percentages of integrin-αvβ3 positive cells were evaluated by flow cytometry in various tumor cell lines and their drug-resistant variants: K029-CDDPR; cisplatin-resistant variant of K029, A375-BRAFIR; BRAF inhibitor-resistant variant of A375 human melanoma cells, MCF7-HER2IR; Her2 inhibitor (Herceptin)-resistant variant of MCF7 human breast cancer cells, Hep3B-SorafenibR; multi-kinase inhibitor sorafenib-resistant variant of Hep3B human hepatoma cells, PC3; EGFR kinase inhibitor (gefitinib)-sensitive human lung cancer cells, NCI-H1975; gefitinib-resistant human lung cancer cells, B16-BRAFIR; BRAF inhibitor resistant variant of murine B16.V600E melanoma cells; MC38-EGFRIR or MC38-CPT11R; EGFR inhibitor (AG494) or CPT-11-resistant variant of MC38 murine colon cancer cells. C, the EpCAM+ epithelial tumors isolated from patients with NSCLC who received multiple cycles of chemotherapy (TX) or not (non-TX) were evaluated for integrin-αv expression by flow cytometry. Similar results were observed in 3 experiments.
damage signals in host antitumor immune responses. For this purpose, B16-BRAFIR were injected subcutaneously into C57BL/6 mice or immunodeficient NOD-SCID mice, and treatments with PLX-4720 and anti–integrin-αv mAb were given to each mouse with or without T cell depletion using anti-CD4- and CD8-specific Abs. The antitumor effect mediated by PLX-4720 and anti–integrin-αv mAb was significantly reduced by T cell depletion in wild-type C57BL/6 wild type, but not in NOD-SCID mice (Fig. 3A). Consistent with a key role for adaptive immunity in the generation of long-lived and specific protective immunity, mice that completely eradicated B16-V600E tumors by the PLX-4720 and anti–integrin-αv mAb rejected a subsequent lethal challenge with B16 melanoma but not unrelated MC38 colon carcinoma, suggesting that the integrin-αvβ3 blockade contributed to the induction of immunologic memory responses (Supplementary Fig. S6).

To further define whether ATM regulation of tumor integrin-αvβ3 is responsible for growing tumors in an T cell-dependent manner, C57Bl/6 mice were challenged with B16-BRAFIR transfected with siRNA vectors specific for integrin-β3 or ATM, and PLX-4720 was administered to each group with or without T cell depletion. The tumor suppressive activities of
PLX-4720 were potent against B16-BRAFIR transfected with integrin-αvβ3 or ATM siRNAs, and we confirmed that these siRNAs efficiently suppress integrin-αvβ3 expression (Supplementary Fig. S3B). However, T cell depletion largely abrogated the antitumor effects mediated by integrin-αvβ3 or ATM knockdown (Fig. 3B and Fig. 3C). These results further validate that ATM-mediated regulation of tumor integrin-αvβ3 is responsible for impeding antitumor responses of PLX-4720 by T cell-mediated mechanisms.

We next evaluated whether integrin-αvβ3 affected host immune phenotypes in tumor microenvironments. Anti-integrin-αv mAb therapy decreased the percentage of CD4⁺ cells expressing IL-10 and Foxp3 (Fig. 4A) and increased the frequencies of IL-17⁺ and IFN-γ⁺ IL-17⁺ CD4⁺ T cells, which have been recently identified as key mediators of antitumor activities (Ref. 32; Fig. 4B). The treatments also enriched the frequencies of total and activated CD8⁺ T cells (CD44⁺CD62L⁻) in TDL (Fig. 4C). These activated phenotypes were not observed in splenocytes or nontumor-draining lymphocytes in the treatment with PLX-4720 and RMV-7, indicating that tumor microenvironments trigger immune tolerance in an integrin-αvβ3-dependent manner (data not shown). The treatment with PLX-4720 resulted in high levels of IL-12 and IFN-γ, but showed little capacity to produce IL-10, in TDL isolated from established B16-BRAFIR cells transfected with the ATM or integrin-αvβ3 siRNA (Fig. 4D). In contrast, PLX-4270 or anti-integrin-αv mAb alone had no effect on IFN-γ secretion and Foxp3⁺ Treg cell frequencies in TDL when PLX-4270-sensitive B16-V600E tumors were targeted (Supplementary Fig. S7). These results show that integrin-αvβ3 plays a critical role in suppressing antitumor immunity against therapy-resistant tumors.

**Integrin-αvβ3 on therapy-resistant tumor cells targets dendritic cells to induce immune tolerance**

Because integrin-αvβ3 elicits multiple effects on host immune functions (13–15), we next examined the effect of integrin-αvβ3 on the functions of dendritic cells, which serve as strong antitumor adjuvants (33). We found that murine BMDC manifested a substantial uptake of live therapy-resistant B16-BRAFIR cells, which was mostly abrogated by the integrin-αvβ3 blockade, but the uptake of apoptotic B16-BRAFIR cells was not affected by the integrin-αvβ3 (Fig. 5A). Immunofluorescence microscopy confirmed the uptake of live B16-BRAFIR cells by BMDCs, whereas the naive B16-V600E cells were not recognized (Supplementary Fig. S8). Human monocyte-derived dendritic cells (MoDC) also have the ability to engulf live Jurkat cells transfected with integrin-αvβ3, but not control plasmid (Fig. 5B and C). Moreover, the integrin-β3 knockdown of tumor cells, but not MoDCs, by specific siRNA resulted in impaired DC engulfment of viable integrin-αvβ3-expressing Jurkat cells (Supplementary Fig. S9), suggesting that the tumor-derived integrin-αvβ3 was responsible for triggering DC uptake of viable tumor cells. The live epithelial cells isolated from pleural effusions of NSCLC patients were also engulfed by MoDCs from the same donor in an integrin-αvβ3-dependent manner, whereas apoptotic cells were recognized by MoDCs irrespective of the integrin-αvβ3 blockade (Fig. 5D). Collectively, these results show that tumor-derived integrin-αvβ3 facilitates DC engulfment of viable tumor cells.
Antigen-presenting cells exploit multiple receptors and secreted proteins to affect the clearance of dying cells (34–36). Because RGD sequence in several ligands, such as MFG-E8 and osteopontin (OPN), serves as a target recognized by integrin-αvβ3, we examined the role of RGD motifs in DC recognition of live resistant tumor cells. The inhibition of RGD recognition with cyclic RGD peptide, but not control RGE peptide, partially reduced the DC uptake of live integrin-αvβ3–expressing Jurkat cells (Supplementary Fig. S10). These results showed that integrin-αvβ3 acts on the ligands bearing RGD sequence to promote live tumor cell engulfment. However, the addition of various neutralizing Abs, such as anti-MFG-E8 Ab and anti-OPN mAb, had little inhibitory effects on the live tumor cell phagocytosis (Supplementary Fig. S11).

The uptake of apoptotic cells by phagocytes has been known to influence various immune functions, such as T helper cell polarization, antigen processing, presentation, and the generation of antigen-specific CTL (37). Thus, we next examined the immunogenic consequences of DC engulfment of live therapy-resistant tumor cells. The recognition of live A375-BRAFIR cells, but not apoptotic one, resulted in a marked downregulation of CD11c (Supplementary Fig. S11). The recognition of live A375-BRAFIR cells serve as sentinels to cross-present immunogenic antigens for T cell activation by capturing dying tumor cells, we examined the impact of live tumor cells on cross-presentation of immunogenic targets by DCs. To do so, we utilized B16-V600E engineered to express OVA (B16-V600E.OVA) and C57BL/6 mice harboring a transgenic TCR specific for an MHC class I-restricted OVA (OT-I). The uptake of apoptotic B16-V600E. OVA cells, irrespective of the anti-integrin-αv blockade, enhanced DC stimulation of OT-I transgenic CD8+ T cells. In contrast, the uptake of live B16-V600E.OVA cells resistant to PLX-4720 (B16-V600E.OVA-BRAFIR) resulted in a reduced activation of OVA-specific CD8+ T cells, which was restored by the integrin-αbβ3 blockade (Fig. 5F). Collectively, these findings suggest that therapy-resistant tumor cells compromise CTL responses by facilitating the engulfment of live tumor cells in an integrin-αvβ3–dependent manner.

**Discussion**

Here, we provide the first evidence that therapy-resistant tumors adopt a novel strategy to suppress antitumor immunosurveillance by triggering integrin-αvβ3 on tumor cells. The chronic activation of DNA damage signals mainly mediated by the ATM-chk2 pathway is responsible for upregulating integrin-αvβ3 expression on tumor cells. The tumor-derived integrin-αvβ3 rendered DCs with ability to engulf live tumor cells.
The live cell phagocytosis resulted in reduced immunogenicity and cross-priming of DCs, leading to impairment of tumor-specific adaptive responses. The blockade of integrin-αvβ3 either with specific antibodies or tumor-specific knockdown promoted efficient tumor recognition by the host immune system, thus overcoming the resistant niche created by therapy-resistant tumors. Altogether, these findings show that the ATM-integrin-αvβ3 axis confers drug-resistant tumor cells with the ability to circumvent endogenous immunosurveillance at tumor microenvironments (Fig. 6).

The significance and pathologic relevance for anticancer drug-resistant tumor cells to evade from antitumor immunosurveillance remain unclear in the present study, but we speculate that these cells have evolved to protect themselves from multiple defense mechanisms against tumorigenesis, including endogenous immune systems. Thus, these multifaceted properties of therapy-resistant tumor cells may contribute to further progression and worse prognosis. However, further elucidation is required to prove this hypothesis.
DNA damage checkpoint machineries serve as barriers to tumorigenesis mainly by p53-dependent mechanisms (27). Here, we delineate some unexpected aspects of DNA damage signals on anticancer therapy resistance: they suppress antitumor immunity by inducing integrin-\(\alpha v\beta 3\) on tumor cells in the setting of a chemoresistant niche. Thus, although DNA damage signals are generally linked with p53-dependent regulation to trigger senescence and apoptosis, intrinsic and environmental regulation of therapeutic responses may change the genetic profiles of tumor cells to evade "classical" DNA damage pathways. Indeed, we found that unique genotoxic stress-induced signals are required for triggering integrin-\(\alpha v\beta 3\) on therapy-resistant tumor cells by ATM-dependent, but p53-independent mechanisms (Fig. 2D). Intriguingly, previous reports unveiled novel pathways in which ATM stimulates NF-\(\kappa B\) signaling cascade in response to genotoxic stimuli such as cytotoxic drugs and irradiation (28). Furthermore, these ATM-NF-\(\kappa B\) pathways could mediate cell survival independently of p53-mediated effector mechanisms (38, 39). Because NF-\(\kappa B\) has been established as a critical sentinel linking inflammation with carcinogenic process, it will be of great interest to determine whether ATM-dependent DNA damage responses activated by tumor cells may be associated with the activation of key carcinogenic inflammatory pathways at chemoresistant niche, further promoting tumorigenic and metastatic potential of therapy-resistant tumors.

Another novel mechanism clarified in this study is that integrin-\(\alpha v\beta 3\) on therapy-resistant tumors mediates immune tolerance by facilitating the DC engulfment of live tumor cells. The DC uptake of live tumor cells is mediated by ligands bearing RGD-motif, although MFG-E8 and OPN had little role in this process. These results show that tumor-derived integrin-\(\alpha v\beta 3\) uses a distinct system to recognize live tumors from those used to recognize apoptotic cells, although further studies are needed to identify the specific ligand for the integrin-\(\alpha v\beta 3\). Although live microbes are sensed by DCs through recognition by specialized phagocytic receptors (40), there has been no literature indicating that DCs have the capacity to engulf viable transformed cells. In this regard, integrin-\(\alpha v\beta 3\) induction on therapy-resistant tumors serves as a novel strategy for compromising DC immunogenicity and antitumor immunosurveillance. Whether live tumor cells, like dying cells, are digested and processed via phagolysosomal pathways in DCs remains obscure, although we did not observe digested forms of chemoresistant tumor cells in DCs in long-term in vitro culture or infiltrating into tumors in vivo (unpublished observation). Further studies should address the intracellular dynamics in DCs whereby tumor cells interrupt their cross-priming machineries.

In summary, we identified integrin-\(\alpha v\beta 3\) as an indispensable factor for creating immune evasion systems in a chemoresistant niche. Although recent clinical development of integrin-\(\alpha v\beta 3\) inhibitors produced significant benefits against advanced gliomas (16), it is critical to clarify the optimal conditions in which particular types of anticancer drugs should be combined with integrin-\(\alpha v\beta 3\) blockade (17). As subsets of cytotoxic drugs may be classified as inducers of "immunogenic cell death" (41, 42), it is possible that the therapeutic options for boosting host immunity, including chemotherapy, radiotherapy, and/or immunotherapy, may maximize the antitumor activities of integrin-\(\alpha v\beta 3\) inhibitors in patients with advanced stages of malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Dr. Koichi Tatsumi (National Institute of Radiological Science, Japan) for providing ATM-deficient mice, Dr. David Cherish (UCSD) for providing human integrin-\(\alpha v\) and \(\beta 3\) plasmids, Dr. Glenn Dranoff (Dana-Farber Cancer Institute) for K029 melanoma cells, and Miss Kyoko Hoshina for her assistance with the in vitro immunoblot assays and animal care.

Grant Support

This study is partially supported by a Grant-in-Aid for Scientific Research for Young Scientists (A) (M. Jinushi), a Grant-in-Aid for Scientific Research (A) (H. Yoshiyama), a Grant-in-Aid for Scientific Research for Innovative Areas (M. Jinushi), the Grant for Joint Research Program of the Institute for Genetic Medicine, Hokkaido University (M. Jinushi and H. Yoshiyama), Uehara Memorial Research Awards (M. Jinushi), Takeda Science Foundation (M. Jinushi), the Sumitomo foundation (M. Jinushi), Kowa Life Science Foundation (M. Jinushi) and The Yasuda Medical Foundation (M. Jinushi).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 20, 2011; revised September 19, 2011; accepted October 18, 2011; published OnlineFirst November 17, 2011.

References


ATM-Mediated DNA Damage Signals Mediate Immune Escape through Integrin-αvβ3–Dependent Mechanisms

Masahisa Jinushi, Shigeki Chiba, Muhammad Baghdadi, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-11-2028

Supplementary Material
Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2011/11/17/0008-5472.CAN-11-2028.DC1

Cited articles
This article cites 42 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/1/56.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/72/1/56.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.