Real-time Imaging of α_v Integrin Molecular Dynamics in Osteosarcoma Cells *In Vitro* and *In Vivo*

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Abstract. *α_v Integrin is involved in various steps of cancer metastasis. In this report, we describe real-time imaging of α_v integrin molecular dynamics in human 143B osteosarcoma cells in vitro and in vivo. We first generated osteosarcoma cells expressing α_v integrin green fluorescent protein (GFP) by transfection of an α_v integrin GFP fusion vector (pCMV6-AC-ITGAV-GFP) into 143B cells. Confocal laser-scanning microscopy demonstrated that α_v integrin immunofluorescence staining co-localized with α_v integrin-GFP fluorescence in 143B cells. When α_v integrin-GFP-expressing 143B osteosarcoma cells were seeded on a dish coated with fibronectin, which is bound by α_v integrin, punctate α_v integrin-GFP was observed by confocal laser-scanning microscopy. When the 143B α_v integrin-GFP cells were seeded onto uncoated plastic, α_v integrin-GFP was diffuse within the cells. When α_v integrin-GFP 143B osteosarcoma cells (1×10^6) were orthotopically transplanted into the tibia of nude mice, the cells aligned along the collagen fibers within the tumor and had punctuate expression of α_v integrin-GFP. In the orthotopic model, the invading osteosarcoma cells had punctate α_v integrin-GFP in the muscle tissue at the primary tumor margin. These results show that α_v integrin-GFP enables the imaging of the molecular dynamics of α_v integrin in osteosarcoma cells in vitro and in vivo.*

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Integrins are involved in angiogenesis, tumor growth, and metastasis. For example, increased expression of β1 integrin is associated with poor prognosis in patients with invasive breast cancer (1), small cell lung cancer (2), and melanoma (3). In our previous study, we knocked down the β1 subunit in human FG red fluorescent protein-expressing (RFP) pancreatic cancer cells using lentiviral-based RNA interference. FG cells are a fast-growing, metastatic variant of the human Colo-357 cell line. Knockdown of β1 integrin reduced primary tumor growth and prevented metastasis in an orthotopic nude-mouse model of pancreatic cancer (4).

In another study, we used the 143B human osteosarcoma cell line, expressing RFP in the cytoplasm and green fluorescent protein (GFP) in the nucleus to seed the lung after *i.v.* injection. A monoclonal antibody to β1 integrin, AIIB2, greatly inhibited the seeding of cancer cells on the lung (experimental metastasis), while a control antibody had no effect, as imaged in real time in the exposed lung in live mice. To image the efficacy of this antibody on spontaneous metastasis, mice with orthotopically-growing 143B-RFP cells in the tibia were treated intraperitoneally with AIIB2 or control antibody to rat IgG1. AIIB2 significantly inhibited spontaneous lung metastasis but not primary tumor growth, possibly due to inhibition of lung seeding of the cancer cells. AIIB2 treatment also increased survival of mice with orthotopically-growing 143B-RFP cells (5).

Since integrins are involved in cancer metastasis, it is important to be able to image the molecular dynamics of integrins during tumor growth and metastasis. In the present study, 143B human osteosarcoma cells were labeled with an α_v integrin-GFP fusion gene in order to visualize the molecular dynamics of α_v integrin-GFP in osteosarcoma cells *in vitro* and *in vivo*.

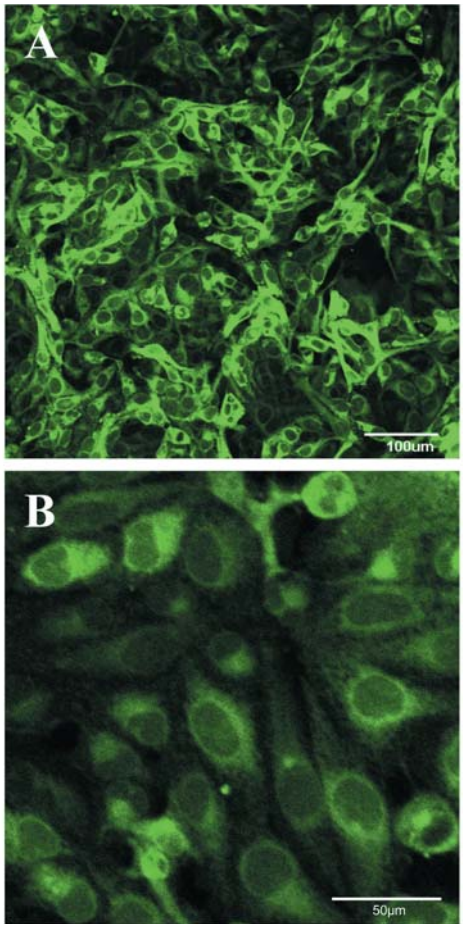

Figure 1. *A: 143B human osteosarcoma cells stably expressing α_v integrin-GFP in vitro. Bar: 100 μm. B: At higher magnification, strong expression of α_v integrin-GFP can be seen around the nuclei of 143B cells. FV1000 confocal microscopy.*

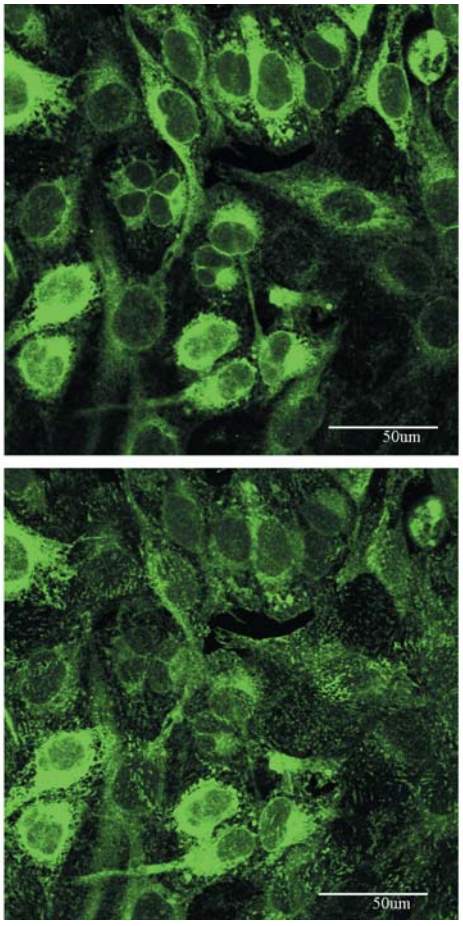

Figure 3. *Visualization of α_v integrin-GFP in 143B cells binding to fibronectin in vitro. Diffuse expression of α_v integrin-GFP was observed in the cytoplasm of 143B cells (left panel). Punctate expression of α_v integrin-GFP was observed at the bottom of the cells binding to fibronectin coated on the plastic cell-culture dish (right panel). FV1000 confocal microscopy.*

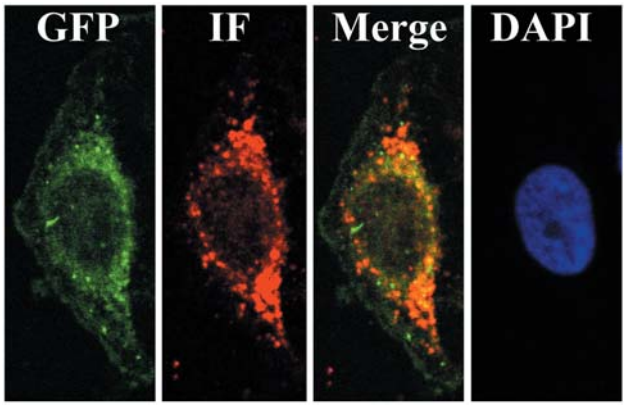

Figure 2. *Co-localization of endogenesis α_v integrin and α_v integrin-GFP in a 143B cell. Immunofluorescence (IF) staining was used to localize endogenous α_v integrin. Please see Materials and Methods for details. Nuclei are indicated by blue staining (DAPI). FV1000 confocal microscopy.*

Materials and Methods

Cells. The 143B human osteosacoma cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA, USA) containing 10% fetal bovine serum (FBS) (Omega Scientific, San Diego, CA, USA) and 1% penicillin/streptomycin at 37˚C in a humidified incubator with 5% CO_2.

Establishment of human osteosarcoma cells expressing α_v integrin-GFP. The pCMV6-AC-ITGAV-GFP vector, containing a fusion gene of α_v integrin and GFP, was obtained from OriGene Technologies (Rockville, MD, USA). 143B cells were seeded at 1×10^6 per 100-mm dish. At 80% confluency, the cells were transfected with pCMV6-AC-ITGAV-GFP using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. After transfection, stably expressing

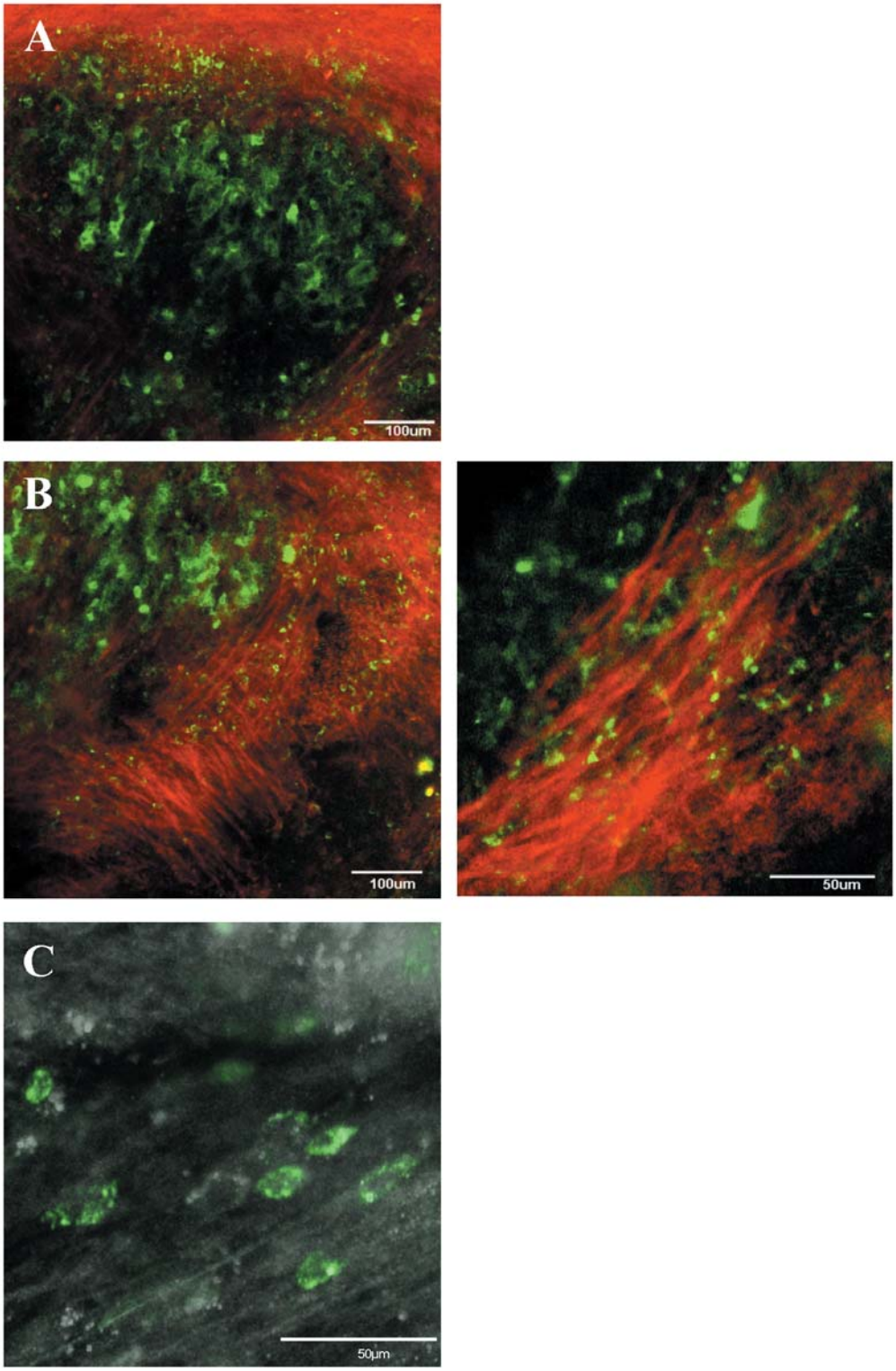

Figure 4. *Visualization of α_v integrin-GFP expression in osteosarcoma cells growing orthotopically in the tibia of nude mice. Green indicates osteosarcoma cells expressing α_v integrin GFP; red indicates collagen fibers, and white indicates autofluorescence of muscle tissue. A: Visualization of the primary tumor. B: 143B osteosarcoma cells at the tumor margin were shrunken and deformed compared to those cells in the center of the tumor (left panel). 143B osteosarcoma cells with punctate α_v integrin-GFP were aligned along collagen fibers (right panel). C: Invading 143B osteosarcoma cells had punctate α_v integrin-GFP expression in the muscle tissue surrounding the primary tumor. FV1000 confocal microscopy.*

cells were selected with G418 (800 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) starting at 24 h after transfection. Stable colonies were then selected in RPMI 1640 medium containing 10% FBS and 500 μg/ml G418 (Sigma-Aldrich) (6).

Immunofluorescence staining. Immunofluorescence staining was performed based on the protocol provided by Cell Signaling Technology (Danvers, MA, USA). The cells were seeded on two-well glass chamber slides (Thermo-Fisher Scientific, Pittsburgh, PA, USA) the day before immunofluorescence staining. The cells were fixed in 4% paraformaldehyde for 10 min. After washing with phosphate buffered saline (PBS) (Omega Scientific), the cells were incubated in blocking buffer containing 5% normal goat serum and 0.3% Triton X-100 for 60 min. The cells were then incubated with a 1:250 dilution of primary antibody (CD51) (Invitrogen) overnight at 4˚C. After washing with PBS, the cells were stained with a 1:200 dilution of secondary antibody (Alexa 555; Invitrogen) for 2 h, followed by staining with 4'6'-diamidino-2-phenylindole (DAPI) (Invitrogen) and imaged by confocal laser-scanning microscopy (FV1000, Olympus, Tokyo, Japan).

Mice. Nude *nu/nu* mice (AntiCancer Inc, San Diego, CA, USA) were bred and housed in a barrier facility. 143B cells (1×10^6), stably expressing α_v integrin-GFP, were orthotopically transplanted into the tibia of 4- to 5-week-old nude mice as previously described (7). Mice were anesthetized with a ketamine mixture (Butler-Schein, Dublin, OH, USA) (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 10 μl H_2O) *via s.c.* injection. All mouse studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under Assurance Number A3873-1.

Real-time in vivo imaging of 143B cells expressing α_v integrin-GFP cells in live mice. Tissue samples and mice were imaged with an Fluoview (FV)1000 (Olympus, Tokyo, Japan) confocal laser-scanning microscope with an XLUMPLFL 20× (0.95 NA) water-immersion objective (8). DAPI was excited at 405 nm, GFP at 488 nm, and Alexa 555 at 543 nm. Collagen fibers were imaged simultaneously in reflectance mode at 488 nm. A skin incision was made to expose the orthotopic tumor before the mouse was positioned on the FV1000 under ketamine anesthesia. Images were analyzed with FV10-ASW Fluoview software (Olympus) and ImageJ (8) and were not modified beyond the standard adjustment of intensity levels.

Results

143B cells expressing α_v integrin-GFP. 143B α_v integrin-GFP cells have a strikingly bright GFP fluorescence in the cytoplasm *in vitro* (Figure 1A), which particularly accumulated around the nuclei (Figure 1B).

Co-localization of α_v integrin-GFP and endogenous α_v integrin in 143B cells. To determine whether localization of transduced α_v integrin-GFP is consistent with that of endogenous α_v integrin in 143B cells, immunofluorescence staining was conducted. As shown in Figure 2, confocal laser-scanning microscopy demonstrated that α_v integrin-GFP co-localized with endogenous α_v integrin.

Imaging of α_v integrin-GFP molecular dynamics in 143B cells growing on fibronectin-coated dishes in vitro. Fibronectin is an extracellular ligand binding to α_v integrin (9). When 143B α_v integrin-GFP cells were seeded on a fibronectin-coated dish (BD Pharmingen, San Diego, CA), although diffuse expression of α_v integrin-GFP was observed in the center of the cell (Figure 3), punctate expression of α_v integrin-GFP was observed interacting with fibronectin coated on the culture dish (Figure 3).

Visualization of the α_v integrin-GFP molecular dynamics in 143B cells growing orthotopically in the nude mouse tibia. 143B α_v integrin-GFP cells were transplanted into the tibia of nude mice. Three weeks after transplantation, the tumor was examined by confocal laser-scanning microscopy *via* a skin flap over the tumor. 143B cells, diffusely or punctately expressing α_v integrin-GFP, were observed in the tumor (Figure 4A). The cells at the tumor margin were shrunken and deformed (Figure 4B) and aligned along collagen fibers, with punctate expression of α_v integrin-GFP (Figure 4B). Invading osteosarcoma cells with punctate α_v integrin-GFP expression were observed in the muscle tissue around the primary tumor (Figure 4C).

Discussion

There have been some previous studies using linking of GFP to proteins of interest in order to image their molecular dynamics. For example, it was found that the onset of CD41-GFP expression in hematopoietic cells was quickly followed by their entry into the circulation (10).

In another study, protrusions of U2OS osteosarcoma cells, expressing a promoter-truncated EGFP–paxillin gene, were observed to undergo cycles of extension and adhesion in collagen gels. These observations demonstrated that molecular dynamics could be imaged with GFP in live osteosarcoma cells (11).

In the current study, the use of fluorescence imaging enabled the observation of α_v integrin molecular dynamics in 143B human osteosarcoma cells, *in vitro* and *in vivo*. Punctate α_v integrin was readily visualized without any immunostaining procedure under confocal laser-scanning microscopy when the 143B α_v integrin-GFP cells were seeded on culture dishes coated with fibronectin, which is a ligand of α_v integrin (9). We also observed the expression of α_v integrin in 143B cells in a primary bone tumor in live mice. Furthermore, using reflectance mode imaging (11), we were able to observe the interaction between collagen fibers in tumors and the osteosarcoma cells expressing α_v integrin at the single-cell level in live mice. At the tumor margin, osteosarcoma cells were shrunken and aligned along collagen fibers, possibly acting as a scaffold for invasion (12).

The molecular imaging model of osteosarcoma cells expressing α_v integrin GFP described in the present study should be a powerful tool for investigating the role of α_v integrin in cancer progression *in vivo*, as well as *in vitro*. The current study can serve as a model to visualize the molecular dynamics of other proteins involved in tumor growth and metastasis using powerful imaging methods of fluorescent protein expression (13-18).

Conflict of Interest

None of the Authors have any conflict of interest in regard to this study.

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