Real-time Study of E-Cadherin and Membrane Dynamics in Living Animals: Implications for Disease Modeling and Drug Development

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Abstract

The ability of tumor cells to invade and metastasize requires deregulation of interactions with adjacent cells and the extracellular matrix. A major challenge of cancer biology is to observe the dynamics of the proteins involved in this process in their functional and physiologic context. Here, for the first time, we have used photobleaching and photoactivation to compare the mobility of cell adhesion and plasma membrane probes in vitro and in tumors grown in mice (in vivo). We find differences between in vitro and in vivo recovery dynamics of two key molecules, the tumor suppressor E-cadherin and the membrane-targeting sequence of H-Ras. Our data show that E-cadherin dynamics are significantly faster in vivo compared with cultured cells, that the ratio of E-cadherin stabilized in cell-cell junctions is significantly higher in vivo, and that E-cadherin mobility correlates with cell migration. Moreover, quantitative imaging has allowed us to assess the effects of therapeutic intervention on E-cadherin dynamics using dasatinib, a clinically approved Src inhibitor, and show clear differences in the efficacy of drug treatment in vivo. Our results show for the first time the utility of photobleaching and photoactivation in the analysis of dynamic biomarkers in living animals. Furthermore, this work highlights critical differences in molecular dynamics in vitro and in vivo, which have important implications for the use of cultured disease models as surrogates for living tissue. [Cancer Res 2009;69(7):2714–9]

Introduction

Animal models have rapidly become essential tools in cancer research; from the determination of basic biological mechanisms to the study of complex human diseases. The analysis of molecular dynamics in an intact host environment, however, remains a major challenge. Fluorescence microscopy has been used to probe molecular dynamics of key proteins within two-dimensional cell cultures (1, 2), but in vivo conditions are more restrictive (3–5), and it is unclear to what extent these in vitro techniques can be applied in vivo. It is therefore important and necessary to develop techniques for the quantification of molecular dynamics in vivo.

Cell-cell interactions mediated by E-cadherin are central to maintaining normal tissue epithelial architecture and have been studied in great detail in vitro (6). The disruption and deregulation of E-cadherin–mediated cell-cell adhesions in cancer is a critical initiation step in the epithelial to mesenchymal transition associated with an invasive phenotype (7). Alterations in E-cadherin dynamics could therefore serve as an early molecular biomarker of metastasis. In order to study E-cadherin and membrane dynamics in their physiologic context, we have applied two complimentary techniques, photobleaching and photoactivation of fluorescence, to visualize protein dynamics in cell-cell junctions and the plasma membrane in tumor xenografts (8–10). Photobleaching is frequently used for the characterization of molecular dynamics within cultured cells. In the simplest approach, the fluorescence within a small region of the sample is bleached using a laser and the recovery of fluorescence into the bleached region is measured over time (11). Photoactivation is a related technique, in which a nonfluorescent (caged) precursor becomes fluorescent upon activation (10). In both cases, two basic variables are derived, (a) the half-time of recovery, an indication of the rate at which probes move in or out of the analysis region, and (b) the immobile fraction, an indication of how much of the probe remains trapped and unable to move out of the analyzed region. Using this approach, we have successfully quantified E-cadherin dynamics in a three-dimensional host setting and provided a molecular readout of the early mobilization events in tumor cells. Our results highlight the necessity and advantages of live animal imaging in the study of tumor development and metastasis, and show that photobleaching and photoactivation can be used in vivo for the quantification of drug action in the treatment of cancer.

Materials and Methods

Plasmids. GFP-E-cadherin and PAGFP were kind gifts from Jennifer Stow (Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, Australia) and Jennifer Lippincott-Schwartz (Cell Biology and Metabolism Branch, NIH, Bethesda, MD), respectively. PAGFP-Farn2Palm was made by substituting GFP in the pEGFP-N1 vector with PAGFP-Farn2Palm (for cloning, see Supplementary Materials).

Cell lines. A431 cells were obtained from (American Type Culture Collection), and maintained as described. The cell-derived matrix was generated as described previously (12). Cells (2 × 10⁴) were plated onto the cell-derived matrix and cells within colonies or migrating cells were targeted for photobleaching and analyzed as described in the Supplementary Materials.

Photoactivation, photobleaching, and analysis in vitro and in vivo. A detailed description of these techniques and analysis, as well as drug treatment in vitro and in vivo, can also be found online in the Supplementary Materials.

Results and Discussion

Photobleaching of GFP-E-cadherin dynamics in vitro correlates with cell migration. Cell-cell adhesion turnover is thought...
to play a key role in cell migration and invasion (6, 7). We therefore used photobleaching to assess whether we could quantify differences in E-cadherin dynamics between stationary and migrating cells. A431 carcinoma cells were plated on cell-derived matrix, and cell junctions between collectively migrating or confluent nonmigrating cells were targeted for bleaching (refs. 13, 14; Fig. 1A and B; Supplementary Movies 1 and 2, respectively). Photobleaching recovery curves from migrating or stationary cells were pooled and analyzed (Fig. 1C). The half-time of GFP-E-cadherin recovery was reduced by 16 seconds in the junctions of cells restrained in colonies (Fig. 1D), and the fraction of GFP-E-cadherin stabilized in cell-cell junctions was 58% higher in confluent cells compared with migrating cells (47.4 ± 1.4% for confluent compared with 30.0 ± 1.5% for migrating cells; Fig. 1D). Similar results were obtained comparing mobile cells at the leading edge of a monolayer wound with confluent nonmigrating cells at the rear of the wound (data not shown). The higher level of GFP-E-cadherin trapped at the cell-cell junctions of stationary cells shows a correlation between cell migration and the level of GFP-E-cadherin trapped within cell-cell junctions. We therefore wanted to assess whether the immobile fraction of GFP-E-cadherin could be measured in vivo and serve as a biomarker for migration potential.

**Photobleaching of GFP-E-cadherin shows fundamental differences in E-cadherin mobility in vivo.** To examine...
GFP-E-cadherin dynamics in vivo, cells were grown as subcutaneous tumors in nude mice and compared with GFP-E-cadherin in the junctions of confluent cells cultured in vitro. In both cases, GFP-E-cadherin was similarly localized to the cell periphery (Fig. 2A and B). Cell-cell junctions could be imaged up to ~70 µm deep in tumor tissue and effectively bleached at ~20 µm, which enabled cells completely surrounded by others to be targeted for bleaching, analogous to the confluent cells targeted in vitro. Initial observation of time-lapse movies suggested that the rate of recovery was much faster in vivo than in vitro. Analysis of pooled recovery curves (Fig. 2C) confirmed that the half-time of recovery was four times faster in vivo (15.3 ± 1.2 seconds in vivo compared with 60.5 ± 2.5 seconds in vitro; Fig. 2D). Furthermore, the fraction of GFP-E-cadherin stabilized in cell-cell junctions was 1.5 times greater in vivo (Fig. 2D). These results show that the mobility of the tumor suppressor E-cadherin, a key protein implicated in the early stages of invasion, is substantially different in vitro and in vivo. Moreover, these differences have profound implications for cancer research, in which the behavior of E-cadherin in cultured disease models is used to elucidate basic biological mechanisms which are extrapolated back into human disease (6, 7).

**Photoactivation shows fundamental differences in plasma membrane dynamics in vivo.** Protein mobility within the plasma membrane also plays a fundamentally important role during cell migration (12, 15). We therefore examined whether plasma membrane dynamics might also differ in the intact host environment compared with cell culture models. To answer this question, we used photoactivatable GFP anchored to the plasma membrane through the farnesylated and doubly palmitoylated membrane targeting sequence of H-Ras (PAGFP-Farn2Palm; ref. 16). Cells were stably transfected with the membrane probe, cultured in vitro or in vivo, and targeted for photoactivation. In vitro, the activated fluorescence was observed to undergo rapid and virtually complete lateral diffusion (Fig. 3A; Supplementary Movie 5). However, initial observations in vivo suggested

![Figure 2](image-url). Photobleaching of GFP-E-cadherin in vitro vs. in vivo. GFP-E-cadherin localization in vitro (A) and in vivo (B). C, fluorescence recovery curves following photobleaching of GFP-E-cadherin performed in vitro (blue) and in vivo (red). D, graphs comparing recovery rates and the amount of GFP-E-cadherin trapped in junctions in vitro (n = 12) and in vivo (n = 15). Columns, mean; bars, SE (bar, 20 µm).
that like E-cadherin, a significant proportion of the membrane probe remained trapped in the site of activation (Fig. 3B; Supplementary Movie 6). Analysis of pooled PAGFP-Farn2Palm decay curves (Fig. 3C) revealed a similar half-time of recovery in vitro and in vivo (Fig. 3D). However, the immobile fraction of the membrane probe was more than five times greater in vivo than in vitro (40.00 ± 4.1% in vivo compared with 7.5 ± 6.5% in vitro; Fig. 3D). Thus, in a similar manner to E-cadherin, we found a substantial increase in the fraction of the membrane probe trapped in vivo compared with in vitro (compare Figs. 2D and 3D). The altered dynamics of both E-cadherin and the plasma membrane in vivo may occur as a result of different environmental cues within the host such as interactions with stromal cells and the extracellular matrix and are currently under investigation.

Dasatinib treatment enhances the stability of E-cadherin in cell-cell junctions in vivo but not in vitro. Drug discovery involves the validation of drugs and targets in vitro to be used for therapeutic intervention in vivo. The high attrition rates of compounds entering clinical trials (17) suggests that drug discovery may be particularly sensitive to the kinds of differences in molecular dynamics in vitro and in vivo that we have observed here for E-cadherin and the membrane probe. We therefore addressed whether photobleaching could be used to

Figure 3. Photoactivation of membrane probe in vitro vs. in vivo. A and B, representative time series demonstrating in vitro and in vivo photoactivation of PAGFP-Farn2Palm. C, fluorescence decay curves following photoactivation of membrane probe in vitro (blue) and in vivo (red). D, graph comparing the rate of fluorescence loss and the amount of membrane probe trapped in the membrane in vitro (n = 40) and in vivo (n = 11). Columns, mean; bars, SE (bar, 10 μm).
compare the effects of pharmacologic intervention in vitro with in vivo. This approach could provide valuable in vivo preclinical data for validating mechanisms of action and guiding dosing regimes. We selected dasatinib, a dual Src/Abl tyrosine kinase inhibitor under consideration for use as an anti-invasive drug in epithelial tumors (18). Although the mechanism of action is not fully understood, it may include effects on E-cadherin dynamics known to be regulated by Src tyrosine kinase activity (19, 20).

Cells were cultured in vitro and in vivo, and treated with dasatinib (20) prior to photobleaching of GFP-E-cadherin. Dasatinib treatment caused no change in E-cadherin levels (Supplementary Fig. S2); however, photobleaching analysis revealed that dasatinib treatment shortened the rate of GFP-E-cadherin recovery both in vitro and in vivo (Fig. 4A). Remarkably, photobleaching analysis also revealed that drug treatment had no effect on the immobile fraction of E-cadherin in vitro but doubled the fraction of E-cadherin trapped in cell-cell junctions in vivo (Fig. 4B). This striking increase in the immobile fraction of E-cadherin in vivo (from 29.5 ± 4.9% to 65.4 ± 2.3%) is significant in relation to our earlier finding that more GFP-E-cadherin is immobilized in junctions between confluent nonmigrating cells within colonies or at the rear of a wound (Fig. 1D; results not shown). This result lends support to a possible mechanism of dasatinib as an anti-invasive drug through the stabilization of cell-cell adhesion, and would be missed by cell culture–based screening assays typically used in drug discovery.

To assess the specificity of the E-cadherin response to dasatinib treatment, E-cadherin recovery dynamics were compared with membrane recovery dynamics in vitro and in vivo. Dasatinib had no effect on either the half-time of recovery or the immobile fraction of the membrane probe in vitro (results not shown). In vivo, the half-time of recovery was similar for both GFP-E-cadherin and the membrane probe. However, dasatinib treatment had significantly different effects on the immobile fraction of GFP-E-cadherin and the membrane probe in vivo: drug treatment increased the immobile fraction of GFP-E-cadherin by a factor of 2 but decreased the immobile fraction of the membrane probe by a factor of 1.6 (from 40.00 ± 4.1% to 24.7 ± 4.0%; Fig. 4C). This highlights the selectivity of dasatinib for E-cadherin in vivo independent of membrane dynamics.

In summary, we have shown the first use of photobleaching and photoactivation to monitor molecular dynamics in living tumors. Our results show that fundamental cellular properties such as the mobility of cell-cell adhesion components or plasma membrane proteins are different in vitro and in vivo. Photobleaching offers the potential to probe the spatial and temporal dynamics of cell-cell junctions in a context-dependent manner; for example, depending on the proximity to migratory stimuli. Such in vivo analysis enables the quantification of subtle early changes in protein behavior in response to therapeutic intervention, which might be missed using cell culture models. The altered mobility of the membrane targeting sequence of H-Ras in vivo highlights the potential that other membrane-targeted signal transduction molecules may behave differently than expected in vivo. Our data critically highlight a shortcoming of conventional drug discovery, the use of cell culture models which fail to recapitulate the behavior of cells in living organisms, and emphasize the importance of early in vivo analysis.
preclinical testing in the drug discovery pipeline. Finally, the adaptation of these techniques for real-time live animal imaging can be extended to the analysis of other key dynamic biomarkers or oncogenes during the study of cancer progression and should provide an in-depth understanding of molecular behavior in a spatial, temporal, and now, contextual setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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