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Role of EGFR Expression Levels in the Regulation of Integrin Function by EGF

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Abstract

Activation of $\beta 1$ integrins in dormant tumor cells has been linked to metastatic progression, suggesting that therapies designed to maintain $\beta 1$ integrins in an inactive state may be useful in the prevention of metastatic disease. Our earlier studies have demonstrated that EGF regulates the activation state of the $\alpha 5\beta 1$ integrin in EGFR overexpressing tumor cells through an ERK/p90RSK signaling pathway. Activation of this pathway by EGF resulted in the Filamin A dependent inactivation of the $\alpha 5\beta 1$ integrin receptor for fibronectin. The current study was designed to address the role of EGFR overexpression in the regulation of $\alpha 5\beta 1$ integrin activation state by EGF. Lentiviral knockdown of EGFR coupled with limited dilution cloning was used to develop A431 squamous carcinoma cell lines expressing high, moderate and low levels of EGFR. Inactivation of $\alpha 5\beta 1$ integrin by EGF was shown to correlate with both the level of EGFR expression and the extent of p90RSK phosphorylation, but not with the level of ERK phosphorylation, suggesting that high levels of EGFR promote $\alpha 5\beta 1$ integrin inactivation through sustained activation of p90RSK. Treatment of cells with EGFR kinase inhibitor resulted in a reactivation of the integrin which could be reversed with the phosphatase inhibitor, Menadione. Taken together, these findings indicate that p90RSK may function to maintain dormancy in tumor cells expressing high levels of EGFR.

Keywords

Integrin activation; tumor metastasis; EGFR; p90RSK; Filamin A; Tumor dormancy; Menadione; Fibronectin

INTRODUCTION

Managing tumor metastasis by manipulating disseminated tumor cells into a prolonged state of dormancy represents an emerging but largely unexplored approach to therapy. Recent studies have pointed to the importance of the tumor microenvironment, particularly the extracellular matrix in the regulation of dormancy [1]. Several laboratories have shown that the interaction of $\beta 1$ integrins with the fibronectin/collagen matrix is a key event in the release of cells from dormancy into an active proliferative state [2-7]. It follows from these studies that metastatic disease might be managed by developing therapies which target

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cellular proteins controlling the activation state (ligand binding activity) of the $\beta 1$ integrin. Keeping the integrin in an inactive state would prevent the interaction of the $\beta 1$ integrin with the matrix, thereby maintaining disseminated tumor cells in a dormant state. To date, there have been few studies which have defined the mechanisms by which $\beta 1$ integrin activation is regulated in malignant cells. In an earlier study, we showed that the addition of EGF to tumor cell lines expressing high levels of EGFR, resulted in a functional inactivation of the $\alpha 5\beta 1$ integrin receptor [8]. Integrin inactivation was shown to result from a novel EGF-dependent inside-out signaling pathway leading to the ERK/p90RSK dependent phosphorylation of Filamin A. The current study was undertaken to understand the impact of EGFR expression levels on the regulation of $\alpha 5\beta 1$ activation state by EGF.

RESULTS AND DISCUSSION

Squamous carcinoma cells (A431) expressing high levels of EGFR were infected with lentivirus expressing shRNA to EGFR. Control cells were infected with vector alone. Clonal cells lines expressing high, moderate and low levels of EGFR were selected by limiting serial dilution. EGFR levels of clonal cells selected for further analysis are shown in Figure 1A and B. Scanning of western blots indicated that the 9-1 clone expressed EGFR at about 10% of the parental and vector control cells. The 17-6 clone expressed EGFR at 30% of control cells. Consistent with our previous study [8] addition of EGF to A431 parental cells resulted in a dose-dependent decrease in adhesion to fibronectin. Inhibitory effects on adhesion were seen in the presence of picomolar amounts of EGF with half maximal inhibitory effects seen at approximately 3.0 ng/ml EGF (500 pM). EGF dependent loss of adhesion could be prevented by inhibitors of EGFR kinase (AG1478), p90RSK (BI-D1870) and MEK (PD184352) (Figure 1C). This finding is consistent with our earlier report showing that EGF mediated $\beta 1$ integrin inactivation in squamous and colon cancer cell lines was dependent upon an EGFR kinase/ERK/p90RSK/ Filamin A (FLNA) signaling pathway [8]. Similar results have also been demonstrated by Gawecka et al. [9] who showed that the expression of a constitutively active RSK2 promoted the FLNA-dependent inactivation of the $\beta 1$ integrin. In both studies, integrin inactivation resulted from the RSK dependent phosphorylation of FLNA. Phosphorylation of FLNA promotes a conformational change to expose a binding site for the cytoplasmic tail of the integrin $\beta 1$ subunit [10-12]. FLNA binding to integrin prevents the association of the integrin activating molecule, talin, thereby maintaining the integrin in an inactive state [13].

The A431 derived clonal cell lines expressing low, moderate and high levels of EGFR were then compared for their ability to adhere to fibronectin in the presence of EGF. As shown in Figure 2A, the clone expressing high levels of EGFR (vector) exhibited a progressive loss of adhesion in the presence of increasing concentrations of EGF. The inhibitory effect of EGF on adhesion was half maximal at approximately 2.0 ng/ml M EGF, paralleling the inhibition seen in parental cells (compare with Figure 1C). In contrast, the cell line expressing low levels of EGFR (clone 9-1) was only slightly affected by EGF, with 80-85% of the cells adhering to fibronectin in presence of 300 ng/ml (50 nM) EGF. The cells expressing moderate levels of EGFR (clone 17-6) gave an intermediate response to EGF. To characterize the impact of EGFR levels on integrin affinity, each cell line was incubated over substrates coated with increasing concentrations of fibronectin (Figure 2B). In the absence

of EGF, each cell line bound similarly to all doses of fibronectin, suggesting that each cell line contained equivalent amounts of active integrins. In the presence of EGF, however, cells expressing high (vector) and moderate (17-6) levels of EGFR were no longer able to adhere at low fibronectin coating concentrations (2-4 $\mu\text{g/ml}$) consistent with these cells exhibiting a decrease in integrin affinity for ligand in the presence of EGF. In contrast, the cell line expressing low (9-1) amounts of EGFR was better able to adhere to fibronectin. These data indicate that the EGF mediated loss of adhesion is due to a decrease in the activation state of the integrin and that the EGF-dependent decrease in integrin activation state is seen in cells expressing high levels of EGFR. Figure 2C shows a western blot comparing EGFR expression levels in two additional clonal lines, 9-10 and 9-12, which were derived in parallel with the 9-1 line used in this study. Similar to the 9-1 clone, the 9-10 and 9-12 clones each expressed EGFR at approximately 10% of the parental A431 cells (Figure 2D). In addition, both the 9-10 and 9-12 clones were refractory to the inhibitory effects of EGF on cell adhesion when compared with the parental A431 cells (Figure 2E). Similar results were obtained with two additional cancer cell lines, DiFi colorectal cells and MDA-MB-468 breast cancer cells. The DiFi cells express EGFR at high levels equivalent to the A431 cells [14]. The MDA-MB-468 cells express moderate levels of EGFR [15] similar to the 17-6 clonal lines. Consistent with the results obtained with the A431 cells and the 17-6 clone, adhesion of the DiFi and MDA-MB-468 cells to fibronectin was decreased in the presence of EGF. In addition, the inhibitory effects of EGF on MDA-MB-468 cell adhesion was more pronounced at lower levels of substrate fibronectin (compare with Figure 2B).

The effect of EGFR expression levels on EGF signaling to the ERK/p90RSK/FLNA pathway was compared using the clonal cells expressing either high (control shRNA) or low (clone 9-1) amounts of EGFR. As shown in Figure 3A, the kinetics and extent of ERK (primarily p42/ERK2) phosphorylation in response to EGF was similar in both cell lines. In contrast, the EGF dependent phosphorylation of RSK and FLNA was less pronounced in the cells expressing low levels of EGFR. A representative control blot showing total p90RSK and total FLNA is shown in Figure 3B. Scanning of similar blots from 4 experiments indicated that the amount of phospho ERK was similar in both cell lines, suggesting that the amount of EGFR in the cell was not affecting the kinetics or extent of ERK activation (Figure 3C). In contrast, the EGF dependent phosphorylation of p90RSK (Figure 3D) and FLNA (Figure 3E) was significantly decreased at all time points in the cells (clone 9-1) expressing low levels of EGFR. These data suggest that ERK activation in response to EGF is unaffected by the level of EGFR expression (Figure 3C). In contrast, the extent of both RSK and FLNA phosphorylation in response to EGF is significantly reduced in cells expressing low levels of EGFR (Figure 3D and E). These data are consistent with a model where overexpression of EGFR mediates the inhibitory effect of EGF on integrin function through a sustained activation of p90RSK and increased phosphorylation of its downstream effector, FLNA. Many tumors express amplified EGFR and are currently treated with inhibitors of EGFR kinase. Our studies would suggest that in some high EGFR expressing tumors, prevailing levels of EGF within a tissue may help to maintain disseminated tumor cells in a dormant state by inactivating $\alpha 5\beta 1$ integrins through autocrine or paracrine signaling [16-18]. It would then follow that treating these tumors with EGFR kinase inhibitors would result in the reactivation of integrins. As predicted, this result can be seen in

Figure 3F. Treatment of A431 cells with EGF inhibits adhesion by 80%, however adhesion is completely restored when cells are treated with the EGFR kinase inhibitor, AG1478. Thus, treatment of tumors with EGFR kinase inhibitors might be expected to contribute to metastasis by releasing those cells expressing high levels of EGFR from dormancy. In such a scenario, reactivating the p90RSK pathway may drive those cells back into a dormant state by inactivating the integrin. To address this question, the phosphatase inhibitor, Menadione (Vitamin K3), was tested for its ability to inactivate integrins in the presence of EGFR kinase inhibitors. Menadione has been shown to activate ERK and to rescue downstream ERK signaling in the presence of EGFR inhibitors [19,20,21]. As shown in Figure 3F, the addition of Menadione reversed the kinase inhibitor mediated adhesion, consistent with integrins returning to an inactive state. Our findings suggest that in tumor cells expressing high amounts of EGFR, picomolar amounts of EGF in the microenvironment may promote tumor dormancy through the p90RSK dependent inactivation of the $\alpha 5\beta 1$ integrin. Decreases in the prevailing levels of EGF or the use of EGFR kinase inhibitors may reactivate the integrins thereby triggering a release from dormancy and contributing to metastatic spread. Therefore, therapies designed to promote dormancy through activation of p90RSK may prove useful in managing tumors expressing high levels of EGFR.

MATERIALS AND METHODS

Reagents and Antibodies

Unless indicated otherwise, reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO). EGF was purchased from R&D Systems (Minneapolis, MN). Antibodies to phospho-p44/42 MAPK (Erk1/2), phospho-Ser2152 FLNA, phospho-Ser380 RSK, and total RSK were purchased from Cell Signaling Technology (Danvers, MA). Rabbit antibodies to ERK2 and FAK were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-EGFR antibody was from BD Biosciences. EGFR inhibitor AG1478 was from Enzo Life Sciences (Plymouth Meeting, PA). Human plasma fibronectin was purified as described previously [22]. The vector pLKO.1 (plasmid 10879) and pLKO.1 expressing non-targeting shRNA (plasmid 1864) were obtained from Addgene (Cambridge, MA). pLKO.1 plasmid expressing EGFR shRNA was from Open Biosystems and was purchased from Thermo Scientific (Pittsburgh, PA). The plasmids used to perform the EGFR gene Knockdown were TRCN0000121069 (cell line 9-1) and TRCN0000121204 (cell line 17-6).

Isolation of the A431 Cell Lines Expressing EGFR shRNA

To produce lentiviruses, HEK293FT cells were co-transfected with 1.2 μ g pCMV- R8.91, 0.13 μ g pMD2G/VSV-G, and 1.2 μ g hairpin-pLKO.1 using lipofectamine 2000 (Life Technologies). After 3 days, the medium containing lentiviral particles was collected and stored at -80°C. Human epidermoid A431 carcinoma cells were infected overnight with lentiviral supernatant containing vector with no shRNA (Plasmid pLKO.1) or EGFR shRNA (pLKO.1-EGFR shRNA), or non-targeting shRNA, in the presence of DMEM containing 10% inactivated serum plus 8 μ g/ml polybrene. The following day, medium was replaced with DMEM containing 10% FBS for 24 h. Cells were then trypsinized and placed in selection medium (DMEM containing 10% FBS supplemented with 1.6 μ g/ml puromycin).

Puromycin-resistant clones expressing EGFR shRNA, non-targeting shRNA or vector alone were selected for expansion and used for experiments.

Cell Adhesion Assay and Western Blot

Cell adhesion assays and immunoblotting of cell lysates were performed as previously described [8]. Plates (48 well) were coated with fibronectin blocked with albumin prior to cell seeding. After 1 h, adherent cells were quantified by staining with 0.05% toluidine blue. Pharmacological inhibitors were added to cell suspensions 1 h prior to plating. EGF was added at the time of seeding. Specific times and doses are provided in the figure legends. For western blots, lysates were prepared from EGF treated cells solubilized in sample buffer (0.12 M Tris pH 6.8, 4% SDS, 20% Glycerol, 5% β -mercaptoethanol and 1 mM Na_3VO_4), separated on SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell Bioscience, NH). Membranes were stained with specific antibodies as described previously [8].

Statistical Analysis

Densitometry measurements were performed using ImageJ software. Values are expressed as % of maximal EGF stimulation (60 min) in control cells. Values are means $\pm$ S.E. of 4 separate experiments. Statistical analysis to determine significance was performed using Student's t test. *, $p < 0.05$. **, $p < 0.01$, ***, $p < 0.001$.

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Figure 1.

Characterization of clonal cell lines expressing varying amounts of EGFR. (A) Lysates from parental cells (A431), clonal cells expressing only pLK0.1 (vector) or EGFR shRNA (9-1; 17-6) were immunoblotted for EGFR and FAK. FAK staining served as a loading control. (B) The EGFR bands shown in (A) were quantified using ImageJ and normalized to FAK. (C) A431 parental cells were allowed to adhere to fibronectin-coated wells in the indicated concentration of EGF for 1 h. In some cases, cells were preincubated with the EGFR kinase inhibitor AG1478, the MEK inhibitor PD184352, and the p90RSK inhibitor BI-D1870 at the indicated concentrations.

Figure 2.

Effect of EGFR expression on integrin activation state. (A, E) Clonal cell lines expressing varying amounts of EGFR were seeded onto fibronectin-coated (10 μg/ml) wells in the indicated concentrations of EGF for 1 h. (B) Clonal cell lines were allowed to adhere for 1 h to wells coated with increasing concentrations of fibronectin in the absence or presence of EGF (3 nM; 18 ng/ml). Cell adhesion was quantified by staining with Toluidine Blue. Error bars indicate S.E. of triplicate samples. (C, D) Lysates from clonal lines were analyzed as described above (Figure 1). (F) DiFi colon cancer cells and MDA-MB-468 breast cancer cells were seeded onto wells coated with the designated amount of fibronectin in the presence or absence of EGF (3 nM; 18 ng/ml).

Figure 3.

Effect of EGFR expression on EGF signaling. (A, B) Clonal cell lines expressing a high (control shRNA) or a low level of EGFR (9-1) were stimulated with EGF (3 nM; 18 ng/ml) for the indicated times. Cell lysates were analyzed by Western blotting for the phosphorylation of ERK1/2, p90RSK, and FLNA. Staining for total FAK served as loading control. (B) A representative blot stained for phospho- and total RSK and Filamin A. (C, D and E) The bands shown in (A) were quantified to evaluate the levels of phospho-ERK (B), phospho-Ser380 p90RSK (C) and phospho-Ser2152-FLNA (D), respectively, and normalized to FAK. Black bars correspond to “control” cells, and white bars to 9-1 cells. (E) Control cells expressing high levels of EGFR were treated with either EGF alone or EGF plus the EGFR kinase inhibitor AG1478 in the presence or absence of increasing amounts of the phosphatase inhibitor Menadione. Error bars indicate S.E. of triplicate samples.