Cell Migration Induced by Native Type IV Collagen Requires PI3K/Akt2 and EGFR Activity in MDA-MB-231 Breast Cancer Cells

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Abstract: Basement membrane (BM) is a specialized extracellular matrix (ECM) that separates epithelial cells from surrounding stroma and regulates various biological processes including morphology, growth, differentiation, adhesion and motility. Type IV collagen is the major component of BM, provides structural framework of all BMs and interacts with cell surface receptors including integrins and discoidin domain receptors (DDR). The DDRs are receptor tyrosine kinases that get activated by collagens in their native triple-helical form and present sustained and slow activation kinetics. Particularly, DDR1 signaling mediates differentiation, immune response, migration and wound healing. However, the signal transduction pathways involved in cell migration induced by native IV collagen in breast cancer cells has been poorly studied. Here we demonstrate that native type IV collagen induces Akt2 and FAK activation through a DDR1, PI3K and epidermal growth factor receptor (EGFR)-dependent pathway in MDA-MB-231 breast cancer cells. In addition, cell migration induced by native type IV collagen requires PI3K, Akt2 and EGFR activity, whereas collagen IV also induces NFκB-DNA binding activity through a DDR1, PI3K, Akt2 and EGFR-dependent pathway. In summary, we demonstrate that migration induced by native type IV collagen requires PI3K/Akt2 and EGFR activity in MDA-MB-231 breast cancer cells.

Keywords: Collagen IV, DDR1, Akt2, PI3K, EGFR, Breast Cancer

1. Introduction

The extracellular matrix (ECM), including the basement membrane (BM), is a network of interacting molecules including collagens, fibronectin and laminin, which stabilizes the physical structures of tissues and regulates several cell processes [1]. Tumor invasion and metastasis require ECM degradation and tissue remodeling, whereas the ability to degrade and penetrate BM has been related with an increased potential for metastasis [2, 3]. BM is a continuous deposit that separates epithelial cells from the stroma, and it regulates cell morphology, growth, differentiation and promotes adhesion and motility of normal and transformed cells. The main component of BM is the type IV collagen, which provides structural framework of all BMs and interacts with cell surface molecules including integrins, proteoglycans and discoidin domain receptors (DDR) [4-6].

DDR1 and DDR2 are receptor tyrosine kinases (RTKs) characterized for an extracellular domain of strong homology to the *Dictyostelium discoideum* protein discoidin that bind to and get activated by collagens in its native triple-helical form. In solid tissues, DDR1 expression is restricted to epithelial cells and is activated by collagens type I to VI and type VIII, whereas DDR2 is restricted to mesenchymal cells and is activated by collagens including type I, III and X [6, 7]. DDRs have a slow activation process compared to other RTKs, and they require hours to achieve maximal tyrosine phosphorylation and then their activation, which is sustained for several days. Particularly, DDR1 signaling mediates differentiation, immune response, migration, wound healing and cancer progression [6, 8-10].

The phosphatidyl inositol 3-kinase (PI3K)/Akt (protein

kinase B) signaling pathway plays an essential role in several cell processes including proliferation, growth, survival, angiogenesis and cancer [11, 12]. The PI3K family consists of three classes of lipid kinases composed of one catalytic and one regulatory subunit, which phosphorylates the 3'-hydroxyl group of phosphatidylinositols and generate molecules that act as second messengers in a number of intracellular signaling pathways, including the activation of PDK and Akt [13]. The class IA PI3Ks are the most widely implicated in cancer, with catalytic subunits that comprise p110 α , p110 β , p110 γ and p110 δ , which have the capability of binding to their regulatory subunits p85 α , p85 β and p55 γ [14, 15]. The Akt family of serine-threonine kinases is the primary downstream mediator of PI3K, and has three isoforms, namely Akt1, Akt2 and Akt3, which share a similar domain structure with an N-terminal pleckstrin homology (PH) domain, a central kinase domain and one C-terminal domain that contains a hydrophobic domain (HD). Akt maximal activation requires the phosphorylation at two sites, one in the kinase domain (Thr-308, Thr-309, Thr-305 in Akt1, Akt2 and Akt3, respectively), and another in the C-terminal domain (Ser-473, Ser-474, Ser-472 in Akt1, Akt2 and Akt3, respectively) [11, 12, 16].

In the present study we demonstrate, for the first time, that native type IV collagen induces Akt2 and FAK activation through a DDR1, PI3K and epidermal growth factor receptor (EGFR)-dependent pathway in MDA-MB-231 breast cancer cells. In addition, we demonstrate that cell migration induced by native type IV collagen requires PI3K, Akt2 and EGFR activity, whereas collagen IV also induces NF κ B-DNA binding activity through a DDR1, PI3K, Akt2 and EGFR-dependent pathway.

2. Materials and Methods

2.1. Materials

Focal adhesion kinase (FAK) antibody (Ab) C-20, Akt2 Ab (F-7), Akt1 Ab (5C10), DDR1 Ab (H-126), phosphospecific Ab to tyrosine PY20 and imatinib mesylate were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Wortmannin and AG1478 were obtained from Calbiochem-Merck Millipore (Darmstadt, Germany). A6730 compound was obtained from Sigma-Aldrich Co. (St. Louis, MO). Phosphospecific Ab to Ser-473 of Akt (anti-p-Akt Ab) was obtained from Cell Signaling (Danvers, MA). Phosphospecific Ab to Tyr-397 of FAK (anti-p-FAK Ab) was obtained from Thermo Fisher Scientific Inc. (NYSE:TMO). Anti- α -actin Ab was kindly provided by Dr. Manuel Hernandez (Cinvestav-IPN, Mexico). [γ -³²P] ATP was obtained from Perkin-Elmer (Boston, MA). Micro Bradford protein assay reagent was from Bio-Rad (Hercules, CA). All other reagents used were of the highest grade available.

2.2. Extraction and Purification of Type IV Collagen

Type IV collagen was extracted and purified from human placenta as described previously [17, 18]. Purity of type IV

collagen was verified by SDS-PAGE and Coomassie stain. Our preparation showed a typical pattern of type IV collagen without any contaminants bands.

2.3. Cell Culture

MDA-MB-231 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.7 g/l sodium bicarbonate, 5% fetal bovine serum (FBS) and antibiotics, in a humidified atmosphere containing 5% CO₂ and 95% air at 37 °C. For experimental purposes, cells were starved in DMEM without FBS for 24 h, before treatment with inhibitors and/or native type IV collagen.

2.4. Cell Stimulation

Cell stimulation was performed as described previously [19]. Briefly, confluent cultures were washed with phosphate buffered saline (PBS) and treated with inhibitors and/or with a solution of type IV collagen in DMEM (native type IV collagen) without FBS for the times indicated. Stimulation was terminated by aspirating the medium (conditioned medium), and cells were solubilized in 0.25 ml of ice-cold RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 1.5 mM MgCl₂, 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride).

2.5. Immunoprecipitation

Equal amounts of protein (700 μ g) were immunoprecipitated overnight at 4 °C with protein A-agarose linked to anti-FAK Ab (1 μ g) or anti-Akt2 Ab (1 μ g). Immunoprecipitates were washed three times with RIPA buffer and extracted in 2 x SDS-PAGE sample buffer (200 mM Tris-HCl pH 6.8, 1 mM EDTA, 6% SDS, 4% 2-mercaptoethanol, 10% glycerol) by boiling 5 min and resolved by SDS-PAGE.

2.6. Western Blotting

Proteins were separated by SDS-PAGE using 10% separating gels followed by transfer to nitrocellulose membranes. After transfer, membranes were blocked using 5% non-fat dried milk in phosphate buffered saline (PBS) pH 7.2 / 0.1% Tween 20 (wash buffer), and incubated overnight at 4 °C with primary Ab. The membranes were washed three times with wash buffer and incubated with secondary Ab (horseradish peroxidase-conjugated) (1:5000) for 2 h at 25 °C. After washing three times with wash buffer, the immunoreactive bands were visualized using ECL detection reagent. Autoradiographs were scanned and the labeled bands were quantified using the Image J software (NIH, USA).

2.7. Interference RNA

DDR1 and Akt2 expressions were silenced in MDA-MB-231 cells by using the silencer siRNAs kit from

Santa Cruz Biotechnology, according to the manufacture's guidelines and 0.3 μ M DDR1 and 0.3 μ M Akt2 siRNAs. One control of scramble siRNAs (0.3 μ M) was included according to the manufacture's guidelines.

2.8. Scratch-Wound Assay

MDA-MB-231 cells were grown to confluence on 35-mm culture dishes, starved for 24 h in DMEM, and treated for 2 h with 12 μ M mitomycin C to inhibit proliferation during the experiment. Cultures were scratch-wounded using a sterile 200- μ l pipette tip, washed twice with PBS and re-fed with DMEM without or with inhibitors and native type IV collagen for the times and concentrations indicated. The progress of cell migration into the wound was photographed at 48 h using an inverted microscope coupled to a camera. Each experimental condition was repeated three times. Cell migration was evaluated by quantification of open wound area using the T-scratch image analysis software.

2.9. Preparation of Nuclear Extracts

Nuclear extracts were prepared as described previously [17]. Briefly, confluent cultures of MDA-MB-231 cells were stimulated without or with 7 μ g/ml native type IV collagen for various times. Cells were lysed with 0.1% nonionic detergent Nonidet P40 in Buffer A (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 6 mM MgCl₂, 10 mM NaF, 1 mM Na₃VO₄, 1 mM DTT, 1 mM PMSF), lysates were pelleted at 2,600 rpm for 5 min and resuspended in Buffer B (20 mM HEPES, pH 7.9, 420 mM NaCl, 20% glycerol 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF, 1 mM DTT, 0.2 mM PMSF). Nuclear extracts were recovered by centrifugation at 12,000 rpm for 15 min at 4 °C and the protein level of each sample was determined by the micro Bradford protein assay.

2.10. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared as described above. Double-strand oligonucleotide containing specific binding sites for NF κ B, 5'-AGCTAAGGGACTTTCGCTGGGGACTTTCAGG-3', was used as probe [20]. An amount of 20 pmol of annealed oligonucleotide was labeled with [γ -³²P] ATP using T4 polynucleotide kinase. The ³²P-labeled oligonucleotide probe (~1 ng) was incubated with 7 μ g of nuclear extract in a reaction mixture containing 3 μ g poly (dI-dC), 0.25 M HEPES pH 7.5, 0.6 M KCl, 50 mM MgCl₂, 1 mM EDTA, 7.5 mM DTT, and 9% glycerol for 20 min at 4 °C. One hundred-fold excess of unlabeled specific was used as specific competitor. The samples were fractionated on a 6% polyacrylamide gel in 0.5x Tris borate-EDTA buffer. Gels were dried and analyzed by autoradiography.

2.11. Statistical Analysis

Results are expressed as mean $\pm$ S.D. Data were statistically analyzed using t-student unpaired test, one-way ANOVA and Tukey or Newman-Keuls multiple comparison tests. Statistical probability of P<0.05 was considered significant.

3. Results

3.1. Native Type IV Collagen Induces Akt2 Activation in MDA-MB-231 Cells

DDRs are activated only when collagen is in its native triple helical form. We previously demonstrated that stimulation of MDA-MB-231 cells with 7 μ g/ml type IV collagen in solution (native type IV collagen) induced DDR1 tyrosine phosphorylation (activation) in a time-dependent manner, reaching two peaks of maximum tyrosine phosphorylation at 10 and 25 h of treatment [19]. Therefore, we performed the following experiments by stimulation of MDA-MB-231 cells with 7 μ g/ml type IV collagen in solution (native type IV collagen). First, we further substantiate our previous findings that native type IV collagen induces DDR1 activation, we stimulated MDA-MB-231 cells for 10 and 25 h with 7 μ g/ml type IV collagen in solution and with denatured type IV collagen, which was prepared by heating the native type IV collagen solution at 90°C for 50 min. Cells were lysed and immunoprecipitated (IP) with anti-DDR1 Ab and the immune complexes were analyzed by Western blotting with phosphospecific Ab to tyrosine PY20. Our results showed that native IV collagen induced DDR1 tyrosine phosphorylation, whereas denatured collagen did not induce DDR1 phosphorylation (Fig. 1A).

We examined whether native type IV collagen induces Akt2 activation. Cultures of MDA-MB-231 cells were stimulated with 7 μ g/ml native type IV collagen for various times and lysed. Cell lysates were IP with anti-Akt2 Ab and analyzed by SDS-PAGE followed by Western blotting with anti-p-Akt Ab, which recognizes the phosphorylation on Ser-473 and Ser-474 of Akt1 and Akt2 respectively. As illustrated in Fig. 1B (upper panel), treatment of MDA-MB-231 cells with native type IV collagen induced an increase on Akt2 phosphorylation at Ser-474 (p-Akt2) that reached at maximum between 3 and 6 h of treatment. Western blotting with anti-Akt2 Ab of the same membranes was used as loading control (Fig. 1B, lower panel).

Since, we demonstrated that native type IV collagen induces DDR1 activation [19], and that native type IV collagen induces Akt2 activation in MDA-MB-231 cells. We determined the role of DDR1 on Akt2 activation. The imatinib compound was used to study the role of DDR1 on Akt2 activation. Imatinib mesylate is an inhibitor of c-Abl tyrosine kinase, PDGFR and tyrosine kinases associated with c-kit [21]. However, it has been recently reported that imatinib mesylate used in the range of 30-100 nM concentration is a selective inhibitor of DDR1 kinase activity [22, 23]. MDA-MB-231 cells were treated for 2 h with 100 nM imatinib and stimulated with 7 μ g/ml native type IV collagen for another 24 h, and cells were lysed. Lysates were IP with anti-Akt2 Ab, followed by Western blotting with anti-p-Akt Ab. Our results showed that treatment with imatinib completely inhibit p-Akt2 induced by native type IV collagen (Fig. 1C).

Akt is the primary downstream mediator of PI3K signaling. We determined whether Akt2 activation, given by its phosphorylation at Ser-474, requires PI3K activity.

MDA-MB-231 cells were treated for 2 h with 100 nM wortmannin, an inhibitor used to inhibit PI3K activity in MDA-MB-231 cells [24], and then stimulated with 7 µg/ml native type IV collagen for 24 h. Cells were lysed and lysates were IP with anti-Akt2 Ab, followed by Western blotting with anti-p-Akt Ab. As illustrated in Fig. 1C, p-Akt2 induced by native type IV collagen required PI3K activity.

3.2. Native Type IV Collagen Induces FAK Activation Through a DDR1/PI3K-Dependent Pathway

Since, we demonstrated that native type IV collagen induces migration through a DDR1-dependent pathway in MDA-MB-231 cells [25]. We determined whether native type IV collagen induces FAK activation, because it is implicated in the migration process. MDA-MB-231 cells were treated with 7 µg/ml native type IV collagen for various times and lysed. FAK activation, given by its phosphorylation at Tyr-397 (p-FAK), was analyzed by immunoprecipitation with anti-FAK Ab and Western blotting with anti-p-FAK Ab. As shown in Fig. 2A, treatment of cells with native type IV collagen resulted in a biphasic p-FAK response, with two peaks of phosphorylation at 3 and 12 h of treatment.

Next, we determined whether FAK activation induced by native type IV collagen required DDR1 and PI3K activity. MDA-MB-231 cells were treated for 2 h with 100 nM imatinib or 100 nM wortmannin, stimulated with 7 µg/ml native type IV collagen for 24 h and lysed. FAK activation was analyzed by immunoprecipitation with anti-FAK Ab and Western blotting with anti-p-FAK Ab. As shown in Fig. 2B, FAK activation induced by native type IV collagen required DDR1 and PI3K activity.

3.3. Native Type IV Collagen Mediates Cell Migration Through a PI3K/Akt2-Dependent Pathway

Native type IV collagen induces migration of MDA-MB-231 cells [25]. We determined the role of PI3K and Akt activity on cell migration. Cultures of MDA-MB-231 cells were treated for 2 h with 100 nM wortmannin or 2 µM A6730, which is a selective inhibitor of Akt1/2 activity [26]. Cell cultures were scratch-wounded and treated with 7 µg/ml native type IV collagen for 48 h. Our results showed that native type IV collagen induced migration and that it is dependent on PI3K and Akt1/2 activity in MDA-MB-231 cells (Fig. 3A and B).

Next, we studied the role of Akt2 on cell migration induced by native type IV collagen. Cell migration assays were performed by using scratch wound assays and MDA-MB-231 cells, in which Akt2 expression was knocked down by using siRNA against Akt2 (Fig. 3C), and treated with 7 µg/ml native type IV collagen for 48 h. As shown in Fig. 3D and E, inhibition of Akt2 expression prevented migration induced by native type IV collagen in MDA-MB-231 cells.

3.4. DDR1 Mediates the Increase of NFκB-DNA Binding Activity Induced by Native Type IV Collagen

Since, NFκB is able to be activated by Akt [27]. We

determined whether native type IV collagen induces NFκB-DNA binding activity. Cultures of MDA-MB-231 cells were stimulated for various times with 7 µg/ml native type IV collagen and nuclear extracts were obtained. NFκB-DNA binding activity was analyzed by EMSA using nuclear extracts and a radiolabeled oligonucleotide probe representing a canonical NFκB binding site. As shown in Fig. 4A, treatment of cells with native type IV collagen induced an increase of NFκB-DNA complex formation in a time-dependent manner, with a maximum of binding at 24 h of treatment. The specificity of these complexes was demonstrated by inhibition of binding in the presence of a cold competitor.

Next, we determined whether DDR1 mediates NFκB-DNA binding activity. MDA-MB-231 cells were treated for 2 h with different concentrations of imatinib (22, 33, 60 nM), stimulated with 7 µg/ml native type IV collagen for 24 h, nuclear extracts were obtained and analyzed by EMSA. As illustrated in Fig. 4B, treatment with different concentrations of imatinib inhibited the increase of NFκB-DNA binding activity induced by native type IV collagen. To further substantiate our findings, EMSAs were performed by using nuclear extracts from MDA-MB-231 cells treated with 7 µg/ml native type IV collagen for 24 h, in which DDR1 expression was knocked down by using siRNA DDR1 (Fig. 4C). Our findings showed that inhibition of DDR1 expression inhibited the increase of NFκB-DNA binding activity induced by native type IV collagen (Fig. 4D).

3.5. Role of DDR1 and PI3K/Akt2 on the Increase of NFκB-DNA Binding Activity Induced by Native Type IV Collagen

We studied the role of PI3K/Akt on the increase of NFκB-DNA binding activity induced by native IV collagen. MDA-MB-231 cells were treated for 2 h with 100 nM wortmannin and 2 µM A6730, treated for 24 h with 7 µg/ml native type IV collagen. Nuclear extracts were obtained and EMSAs were performed. As shown in Fig. 4B and D, inhibition of PI3K and Akt1/2 activity inhibited the increase of NFκB-DNA binding activity induced by native type IV collagen.

In order to determine the participation of Akt2, EMSAs were performed by using nuclear extracts from MDA-MB-231 cells treated for 24 h with 7 µg/ml native type IV collagen, in which Akt2 expression was knocked down by using siRNA Akt2 (Fig. 4A). Our findings showed that inhibition of Akt2 expression prevented the increase of NFκB-DNA binding activity induced by native type IV collagen (Fig. 4D).

3.6. Role of EGFR on Cell Migration and NFκB-DNA Binding Activity Induced by Native Type IV Collagen

Since, we previously demonstrated that native type IV collagen induces secretion of gelatinases through an EGFR-dependent pathway in breast cancer cells [19]. We studied the role of EGFR on Akt2 and FAK activation, migration and NFκB-DNA binding activity induced by native

type IV collagen.

In order to determine the role of EGFR on Akt2 and FAK activation induced by native type IV collagen, MDA-MB-231 cells were treated for 2 h with 500 nM AG1478, which is a selective inhibitor of EGFR activity [28], treated with 7 μ g/ml native type IV collagen for 24 h and lysed. Activation was

analyzed by Western blot using anti-p-Akt2 Ab and anti-p-FAK Ab. Our findings showed that treatment with EGFR inhibitor completely blocked the activation of FAK and Akt2 (p-FAK and p-Akt2) induced by native type IV collagen (Fig. 5A and B).

Figure 1. Native type IV collagen induces Akt2 activation through a DDR1 and PI3K-dependent pathway in MDA-MB-231 breast cancer cells. Panel A. MDA-MB-231 cells were treated for 10 and 25 h with 7 μ g/ml type IV collagen in solution and with denatured type IV collagen, which was prepared by heating the native type IV collagen solution at 90 °C for 50 min. Cells were lysed and immunoprecipitated (IP) with anti-DDR1 Ab and the immune complexes were analyzed by Western blotting with phosphospecific Ab to tyrosine PY20. The membranes were analyzed further by Western blotting with anti-DDR1 Ab. Panel B. MDA-MB-231 cells were treated without or with 7 μ g/ml type IV collagen in solution (Col IV) for the indicated times and lysed. Panel C. MDA-MB-231 cells were treated without (-) or with (+) 100 nM imatinib or 100 nM wortmannin (Wort), stimulated with 7 μ g/ml Col IV for 24 h and lysed. Lysates were analyzed by immunoprecipitation (IP) with anti-Akt2 Ab followed by Western blotting with anti-p-Akt Ab. The membranes were analyzed further by Western blotting with anti-Akt2 Ab. The graphs represent the mean $\pm$ S.D. of at least three independent experiments and are expressed as fold of p-Akt2 above untreated cells (Control). Asterisks denote comparisons made to untreated cells. * P <0.05, ** P <0.01, *** P <0.001.

To determine the role of EGFR on cell migration, cultures of MDA-MB-231 cells were treated for 2 h with 500 nM AG1478. Cell cultures were scratch-wounded and treated with 7 μ g/ml native type IV collagen for 48 h. Our results showed that inhibition of EGFR activity inhibited the migration induced by native type IV collagen (Fig 5C and D). To study the role of EGFR on the increase of NF κ B-DNA binding

activity induced by native type IV collagen. MDA-MB-231 cells were treated for 2 h with 500 nM AG1478, treated for 24 h with 7 μ g/ml native type IV collagen, nuclear extracts were obtained and EMSAs were performed. Our findings showed that inhibition of EGFR activity prevented the increase of NF κ B-DNA binding activity induced by native type IV collagen (Fig. 5E).

4. Discussion

In normal conditions, ECM mediates several cell functions including differentiation, polarity and survival. However, during the metastasis process, there is a disruption of physiological interactions of tumor cells with the surrounding ECM, which contributes to migratory and invasive programs [29].

In the normal mammary gland, BM is a continuous deposit

that separates epithelial cells from surrounding stroma, and is the first barrier that cancer cells must traverse to produce metastasis. The integrins and DDRs are the most widely expressed collagen receptors in cells, which activate specific signaling pathways and responses. Particularly, DDR1 is activated by native type IV collagen and does not require the activity of collagen-binding integrins [30]. However the signal transduction pathways induced by activation of DDR1 with type IV collagen has been poorly described.

Figure 2. Native type IV collagen induces FAK activation through a DDR1 and PI3K-dependent pathway. Panel A. MDA-MB-231 cells were treated without or with 7 μ g/ml Col IV for the indicated times and lysed. Panel B. MDA-MB-231 cells were treated without (-) or with (+) 100 nM imatinib or 100 nM Wort, stimulated with 7 μ g/ml Col IV for 24 h and lysed. Lysates were analyzed by IP with anti-FAK Ab followed by Western blotting with anti-p-FAK Ab. The membranes were analyzed further by Western blotting with anti-FAK Ab. The graphs represent the mean $\pm$ S.D. of at least three independent experiments and are expressed as fold of p-FAK above untreated cells. Asterisks denote comparisons made to untreated cells. * P <0.05, ** P <0.01, *** P <0.001.

DDR1 and DDR2 present 15 and 14 tyrosine residues in their cytoplasmic domains respectively, which are phosphorylation sites upon receptor activation by collagens. The tyrosine phosphorylation sites recruit cytoplasmic signaling molecules containing SH2 domains and phosphotyrosine-binding (PTB) domains, which promote assembly of protein complexes able to transduce receptor signaling [6, 10]. Particularly, native type I collagen promotes

the association between the p85 subunit of PI3K with the tyrosine-881 of DDR1 in T47D cells [31]. In line with this notion, our findings demonstrate that native type IV collagen induces Akt2 activation and that it is mediated by DDR1 and PI3K activity in MDA-MB-231 breast cancer cells.

Focal adhesions are the sites where integrins mediate adhesion links to the actin cytoskeleton and the ECM, their components are diverse and include scaffolding proteins,

GTPases and kinases [32, 33].

Figure 3. Role of PI3K/Akt2 on cell migration induced by native type IV collagen. Panel A. MDA-MB-231 cells were grown in 35 mm dishes and treated with 12 μ M mitomycin C for 2 h. Cultures were scratch-wounded, treated with 100 nM Wort or 2 μ M A6730 and stimulated with or without 7 μ g/ml Col IV and pictures were taken at 48 h after wounding. Panel B. The graph is the mean $\pm$ S.D. of at least three independent experiments and is expressed as fold of migration above untreated cells. Panel C. MDA-MB-231 cells were transfected with Akt2 specific or scramble siRNAs. Akt2, Akt1 and actin expression were analyzed by Western blotting with anti-Akt2 Ab, anti-Akt1 Ab and anti-actin Ab respectively. Akt1 and actin expressions were used as loading controls. Panel D. MDA-MB-231 cells were transfected with Akt2 specific or scramble siRNA. Cultures were scratch-wounded and stimulated with 7 μ g/ml Col IV and pictures were taken at 48 h after wounding. Panel E. The graph is the mean $\pm$ S.D. of at least three independent experiments and is expressed as fold of migration above untreated cells. * $P < 0.05$.

FAK is a 125 kDa protein tyrosine kinase localized to focal adhesions that can be activated by multiple agonists including ECM components and growth factors (EGF, HGH and PDGF). Activation of FAK is mediated by its autophosphorylation at Tyr-397, which is the docking site for SH2 domains of a number of signaling proteins including Src, PI3K and Grb7. FAK is implicated in the regulation of cell spreading, migration, differentiation, proliferation, apoptosis, invasion, survival and angiogenesis [34, 35]. We previously

demonstrate that native type IV collagen promotes migration through a DDR1-dependent pathway in MDA-MB-231 cells [25]. In line with this notion, we demonstrate here that native type IV collagen induces FAK activation through a DDR1 and PI3K-dependent pathway in MDA-MB-231 cells. These results are in agreement with a previous report demonstrating that cell migration mediated by FAK requires the activity of PI3K and the formation of FAK-PI3K complex [36].

Figure 4. Role of DDR1 and PI3K/Akt2 on the increase of NFκB-DNA binding activity induced by native type IV collagen. Panel A. MDA-MB-231 cells were stimulated without or with 7 μg/ml Col IV for the indicated times and nuclear extracts were obtained. Panel B. MDA-MB-231 cells were treated with various concentrations of imatinib (22, 33, 60 nM) and 100 nM Wort, stimulated without or with 7 μg/ml Col IV and nuclear extracts were obtained. Panel C. MDA-MB-231 cells were transfected with DDR1 specific or scramble siRNAs. DDR1 and actin expression were analyzed by Western blotting with anti-DDR1 Ab and anti-actin Ab respectively. Actin expression was used as loading control. Panel D. MDA-MB-231 cells were treated with 2 μM A6730 and MDA-MB-231 cells transfected with DDR1 and Akt2 specific or scramble siRNAs were treated with 7 μg/ml Col IV and nuclear extracts were obtained. NFκB-DNA binding activity was analyzed by EMSA. Controls included EMSA reactions with 100-fold excess of cold NFκB probe as competitor. The autoradiographs shown are representative of at least three independent experiments.

The PI3K/Akt pathway regulates a variety of cell processes including, migration, proliferation, survival, protein synthesis, glucose metabolism and carcinogenesis [37, 38]. Particularly, Akt1 plays an important role in tumor development, whereas Akt2 participates in tumor invasion and metastasis in mouse models of oncogene-induced mammary tumorigenesis [39-41]. Here we demonstrate that native type IV collagen induces Akt2 activation, and that migration induced by native type IV collagen requires PI3K and Akt2 activity. We propose that native type IV collagen plays a pivotal role in migration/invasion process through the activation of PI3K and Akt2 in breast cancer cells. Supporting our proposal, we previously demonstrated that migration and invasion induced by arachidonic acid require PI3K and Akt activity in MDA-MB-231 breast cancer cells [42].

Akt phosphorylates a number of proteins, including IκB, which releases NFκB and allows it to translocate to the nucleus and activates target genes, such as those involved in

inflammation, metastasis and apoptosis/survival. The transcription factor NFκB is involved in cancer promotion, because it regulates the expression of many genes, including those involved in clonal expansion, growth, angiogenesis, adhesion, extravasation and degradation of ECM components [43, 44]. Particularly, we previously demonstrated that arachidonic acid induces NFκB-DNA binding activity through a PI3K/Akt-dependent pathway in MDA-MB-231 breast cancer cells [42]. In line with this notion, here we demonstrate that native type IV collagen induces NFκB-DNA binding activity and that it is mediated by DDR1 and PI3K/Akt2 activity in MDA-MB-231 cells. We propose that degradation of BM and release of native type IV collagen induces the expression of genes implicated in the invasion/metastasis process through the activation of NFκB in MDA-MB-231 cells. Supporting our proposal, it has been demonstrated that NFκB mediates expression of tumor promoting genes, such as TNF, IL-1, iNOS and MMP-9 [43].

Figure 5. Role of EGFR on cell migration and NF κ B DNA binding activity induced by native type IV collagen. Panel A and B. MDA-MB-231 cells were treated without (-) or with (+) 500 nM AG1478, stimulated with 7 μ g/ml Col IV for 24 h and lysed. Lysates were analyzed by IP with anti-Akt2 Ab and anti-FAK Ab followed by Western blotting with anti-p-Akt2 Ab and anti-p-FAK Ab respectively. The membranes were analyzed further by Western blotting with anti-Akt2 and anti-FAK Ab respectively. The graphs represent the mean $\pm$ S.D. of at least three independent experiments and are expressed as fold of p-Akt2 or p-FAK above untreated cells. Panel C. MDA-MB-231 cells were grown in 35 mm dishes and treated with 12 μ M mitomycin C for 2 h. Cultures were scratch-wounded, treated with 500 nM AG1478 and stimulated with or without 7 μ g/ml Col IV and pictures were taken at 48 h after wounding. Panel D. The graph is the mean $\pm$ S.D. of at least three independent experiments and is expressed as fold of migration above untreated cells. Panel E. MDA-MB-231 cells were treated with 500 nM AG1478, stimulated without or with 7 μ g/ml Col IV and nuclear extracts were obtained. NF κ B-DNA binding activity was analyzed by EMSA. Controls included EMSA reactions with 100-fold excess of cold NF κ B probe as competitor. The autoradiograph showed is representative of at least three independent experiments.

EGFR and Her-2 overexpression correlates with a reduction in survival and induction of invasion and metastasis in malignant breast cancer [45]. Furthermore, transactivation of EGFR induced by G-protein couple receptors (GPCRs) is a well-documented pathway that occurs via activation of metalloproteinases (MMPs) and subsequent release of epidermal growth factor (EGF)-like ligands, including HB-EGF, from growth factors precursors in the plasma membrane [46, 47]. Particularly, Src family kinases are suggested upstream and downstream mediators of EGFR activation in GPCRs-induced transactivation, where Src kinases play a role in the MMPs activation [46, 48]. In addition, EGFR transactivation is also mediated by ligands

that activate RTKs including growth hormone and PDGF [49-51]. Our findings showed that Akt2 and FAK activation, cell migration and NF κ B-DNA binding activity induced by native type IV collagen require EGFR activity in MDA-MB-231 cells. Since, native type IV collagen promotes Akt2 and FAK activation through a DDR1/PI3K-dependent pathway and that migration and NF κ B-DNA binding activity require DDR1, PI3K and Akt2 activity, we propose that activated DDR1 by native type IV collagen transactivates EGFR and then EGFR mediate PI3K activation, which mediate Akt2 and FAK activation, subsequently Akt2 mediates migration and NF κ B-DNA binding activity in MDA-MB-231 cells.

In conclusion, our findings demonstrate, for the first time, that native type IV collagen promotes FAK and Akt2 activation, which require DDR1, PI3K and EGFR activity in MDA-MB-231 breast cancer cells. Moreover, native type IV collagen induces cell migration and NF κ B-DNA binding activity through a PI3K, Akt2 and EGFR-dependent pathway. Our findings define a new role of EGFR in the signal transduction pathway mediated by DDR1 activated with native type IV collagen in MDA-MB-231 breast cancer cells. Our results suggest that EGFR through PI3K and Akt2 activation play an important role in the migration/invasion process mediated by DDR1 in breast cancer cells.

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