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Density-Dependent ERK MAPK expression regulates MMP-9 and influences growth

Vincent Marchese, Jazmin Juarez, Priyal Patel, and Dorothy Hutter-Lobo*

Biology Department, Monmouth University, 400 Cedar Avenue, West Long Branch, NJ 07764

Abstract

Previous work has shown that expression of the extracellular signal regulated kinase (ERK) is decreased by high density in normal fibroblast cells (BJ), and this was correlated with increased expression of mitogen-activated protein kinase phosphatases (MKPs). Because of these differences in ERK regulation upon contact inhibition, it is likely that other cellular responses may be influenced by the attainment of a contact-inhibited state. Expression of matrix metalloproteinase-9 (MMP-9) and cadherin cleavage were both found to be decreased upon reaching high culture density. Inhibition of ERK activity with the MEK inhibitor PD98059 resulted in increased expression of cadherins, while constitutive activation of ERK through the use of expression of an ERK construct with a D319N sevenmaker mutation resulted in decreased expression of cadherins and enhanced colony formation of HT-1080 fibrosarcoma cells. Taken together, these results corroborate a role for the regulation of ERK upon the attainment of a contact-inhibited state with increased expression of cadherins.

Keywords

MMP-9; contact inhibition; ERK; cadherin

Introduction

Contact inhibition of proliferation is a reversible form of growth control exhibited by normal cells grown in culture [1]. When cultured cells reach a density in which a monolayer is formed, their proliferation is negatively regulated. The transcription of a variety of genes is increased upon contact inhibition, and most likely requires complex coordination of multiple signals, including receptors and cytoskeletal elements [2]. The growth cessation resulting from contact could be from a combination of several factors, including soluble signaling molecules [3], mechanical stress [4], and cell-cell adhesion molecules [5]. In addition to cessation of proliferation, cell-cell contact can also limit movement, causing contact inhibition of locomotion [6], which may also be regulated by similar mechanisms as contact inhibition of proliferation. Mechanical stress can activate gene expression changes that can lead to cytoskeletal rearrangements or migration [7 – 9]. Epidermal growth factor (EGF) is one soluble factor which has been implicated in promoting migration of cancer cells through

*Corresponding author: Dorothy Hutter-Lobo, Ph.D., Biology Department, Monmouth University, 400 Cedar Avenue, West Long Branch, NJ 07764, dhutter@monmouth.edu, 732-263-5546.

downregulation of FAK activity [10]. EGF-mediated chemotaxis has also been implicated in directing migration of rat breast adenocarcinoma cells undergoing contact inhibition of locomotion [11]. Contact inhibition, however, is a reversible process, with proliferation resuming if additional space between cells becomes available (as in wound healing). In contrast, the cessation of proliferation upon senescence is not reversible, suggesting an alternate form of negative regulation of growth during contact inhibition [12]. As transformed and cancerous cells lack contact inhibition of proliferation [13], previous work in this laboratory has investigated potential differences in mitogen-activated protein kinase (MAPK) signaling pathways in normal and transformed cells.

MAPKs, including the c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 families of proteins, allow cells to respond to a variety of growth factor and stress signals. MAPKs are regulated by phosphorylation of conserved threonine and tyrosine residues, and once activated, influence the activity of multiple downstream targets, including transcription factors and other protein kinases, resulting in many diverse cellular responses ranging from proliferation control to apoptosis [14, 15]. Control of MAPK pathways is vital in order to effectively regulate an appropriate cellular response to external stimuli. Such control is moderated by tight regulation of upstream kinases, including members of the MEK family [16]. Negative control is further regulated by phosphatases. One group of phosphatases that has a regulatory role on the MAPK family are the mitogen-activated protein kinase phosphatases (MKPs) – family of at least 10 proteins which are capable of deactivating MAPKs by dephosphorylating both the threonine and tyrosine residues of MAPKs. MKPs are typically activated by the same stimuli, including growth factors and stresses, which activate MAPKs, and serve as feedback regulators of MAPK activity. MKPs vary in their subcellular localization, with some remaining nuclear, and others being cytoplasmic or found in both locations [17]. Previous work in this laboratory has examined the role of MKP regulation of MAPK pathways during regulation of contact inhibition of proliferation. During contact inhibition in normal fibroblasts, activation of ERK declines, along with a concurrent increase in the expression of MKPs [18]. Similarly, activation of p38 was shown to be attenuated upon contact inhibition, while overexpression of a phosphatase-resistant p38 construct enhanced proliferation [19]. The goal of this work was to identify if alterations have been previously seen in MAPK signaling upon contact inhibition may be regulating downstream cellular events necessary for contact inhibition of proliferation or movement. Evidence is presented for the role of MAPK regulation in regulation of cellular adhesion. Matrix metalloproteinase-9 (MMP-9) was downregulated at contact inhibition, correlated with a decrease in activity of ERK. Subconfluent cultures, however, maintained active ERK and had less cleavage of cadherins. Inhibition of ERK activity resulted in less cadherin cleavage, while transfection of fibrosarcoma cells with a phosphatase-resistant ERK construct resulted in increased ability of fibrosarcoma cells to form colonies in soft agar. Colony formation of fibrosarcoma cells was decreased by inhibition of MEK. Taken together, these results indicate that changes in MAPK signaling pathways upon contact inhibition play a role in modulating signaling pathways needed for migration.

Materials and Methods

Cell lines and culture conditions.

Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Complete culture medium was replaced every three- four days to prevent serum-starvation. The BJ cell line, normal human foreskin fibroblasts, and HT-1080 fibrosarcoma cells were purchased from ATCC (Manassas, VA, USA). Culture density was determined by direct counting with a hemocytometer, utilizing trypan blue dye exclusion. Confluent HT-1080 cells (100 mm dish) averaged 14×10^6 cells/dish, while subconfluent HT-1080 cells averaged 4×10^6 cells/dish. Similarly, confluent BJ cells (100 mm dish) averaged 3.4×10^6 cells/dish and subconfluent cultures were 1.7×10^6 cells/dish. To alter MAPK activity, HT-1080 cell cultures were serum-starved overnight prior to treatment with 50 μ M PD98059 for 24 hrs (Promega, Madison, WI, USA) [20] or 300 μ M H₂O₂ (Sigma, St. Louis, MO, USA) for 10 min. preceding western blot analysis.

Antibodies and western blot analysis.

Polyclonal pan-cadherin (H-300) and monoclonal MMP-9 (6-6B) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyclonal ERK1/2 and anti-ACTIVE MAPK were purchased from Promega (Madison, WI, USA). Monoclonal FLAG-M2 and polyclonal actin antibodies were purchased from Sigma (St. Louis, MO, USA). Western blot analysis was performed as previously described [18]. Briefly, cell cultures were washed twice with ice-cold phosphate-buffered saline (PBS). Subconfluent fibroblast cells were lysed in 600 μ L lysis buffer, while confluent fibroblast cells as well as both subconfluent and confluent fibrosarcoma cells were lysed in 1000 μ L of lysis buffer (20 mM N-2-hydroxyethylpiperazine-N¹-2-ethane sulfonic acid (HEPES), pH 7.4, 50 mM β -glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM ethylene bis (oxyethylenenitrilo) tetraacetic acid (EGTA), 1 mM DTT, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 μ M leupeptin, 2 μ M aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were clarified by centrifugation at 14,000 rpm for 10 min. 15 μ g of each protein sample was resolved by electrophoresis through 8% or 10% Tris-glycine-SDS gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and enhanced chemiluminescence reagent (ECL Plus, Amersham, Uppsala, Sweden) was used for the detection of the immunoreactive bands. Densitometry was performed on reactive bands utilizing the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>) and results were normalized to β -actin levels.

Plasmids and transfection.

The construction of the pSR α -FLAG and pSR α -FLAG-ERK2DN (containing the point mutation D319N) vectors have previously been described [21]. For transfection, 1.8×10^6 HT-1080 fibrosarcoma cells were plated into 100 mm dishes and were used for transfection upon reaching approximately 90% confluence. 24 μ g of pSR α -FLAG or pSR α -FLAG-ERK2DN were transfected using Lipofectamine 2000 according to manufacturer's

specifications (Invitrogen, Carlsbad, CA, USA). Transfected cells were assayed using by western blot analysis or used to initiate colony formation assays 48 hrs post-transfection.

Soft agar colony formation assay.

HT-1080 cells transfected with pSR α -FLAG or pSR α -FLAG-ERK2DN for 48 hrs, or treated with PD98059 for 24 hrs. or untreated controls were utilized for a soft agar colony formation assay with slight modifications to the protocol previously described [22]. Briefly, the base agar contained 0.5% agar, 1X DMEM, and 10% fetal bovine serum, and the top agar contained 0.35% agar, 1X DMEM, and 10% fetal bovine serum. In addition, 50 μ M PD98059 was added to the top agar in appropriately treated samples. 1.0×10^4 cells were used per 35-mm plate. Cells were re-feed with complete medium or medium containing 50 μ M PD98059 for 10 days. Colonies were visualized by staining with 0.005% crystal violet for 1 hr. and were destained with water.

Results

Cell confluence and regulation of MMP-9 and cadherins

MMP-9 is a member of the matrix metalloproteinase family, playing a role in the degradation of the extracellular matrix [23], including the cleavage of cadherins [24], promoting the ability for cells to loose adhesion and migrate. To determine the influence of cell confluence on the expression of MMP-9, normal fibroblasts (BJ), which display contact inhibition, and fibrosarcoma cells (HT-1080) were analyzed for MMP-9 expression at subconfluent and confluent states of culture growth. The Coomassie stained membrane and actin detection are shown as loading controls (Fig. 1a). In both cell lines, it was determined that MMP-9 was curtailed upon attainment of a confluent state, suggesting the possibility that downstream targets of MMP-9, including cadherins, may be protected by a confluent state. Densitometric analysis of the MMP-9 bands was performed and statistical analysis of the results were found to be significant in both cell lines (Fig. 1b). To establish a link between ERK activity and MMP-9 expression, fibrosarcoma cells (HT-1080) were treated with the MEK inhibitor PD98059. The inhibition of ERK activity subsequently decreased MMP-9 expression (Fig 2), while conversely, treatment of fibrosarcoma cells (HT-1080) with H₂O₂, which activates ERK [25], resulted in enhanced MMP-9 expression.

In order to investigate the presence of cadherins and any fluctuations in relation to cell confluence, normal fibroblasts (BJ) and fibrosarcoma cells (HT-1080) were examined for cadherin expression in subconfluent and confluent states of growth (Fig. 3). In both cell lines, subconfluent and confluent cultures were found to have expression of cadherins detectable of a size expected of full-length cadherins (120 kDa). In contrast, however, the subconfluent cultures also revealed a fragment of a smaller size, approximately 80 kDa. Though this fragment was present in both cell lines, the expression was more pronounced in the normal fibroblast line, suggesting the possibility that this could be a cleavage product of cadherins resulting from increased MMP-9 expression seen at a subconfluent state. Positive control lysate (A-431) contained detected bands of the same weights, while equivalent loading was demonstrated through actin detection and staining of the membrane with Coomassie blue.

Identifying the influence of the ERK MAPK pathway on cadherin cleavage

Previous work in the laboratory has shown that activation of the ERK MAPK is influenced by contact inhibition, with normal fibroblasts demonstrating reduced ERK activity in a confluent state [18]. The down-regulation of the ERK MAPK pathway upon contact inhibition may therefore be influencing the subsequent down regulation of MMPs or other proteases that may influence cadherin expression. To investigate the potential involvement of ERK1/2 in the formation of cleaved cadherins, ERK1/2 activity was inhibited in fibrosarcoma cells through treatment with the MEK inhibitor PD98059 [26]. Inhibition of MEK led to a decrease in ERK1/2 activity and increased expression of full-length cadherins (Fig. 4), suggesting a role for ERK1/2 in the regulation of cadherin expression.

Constitutive activation of ERK results in decreased expression of cadherins and enhanced growth of colonies in soft agar

To further characterize the relationship of ERK activation to invasiveness of the fibrosarcoma cells, a FLAG-ERK2DN construct was transfected into fibrosarcoma cells. This construct expresses a mutant form of ERK2 (D319N) which renders it unable to be inactivated by dual-specificity protein phosphatases [21, 27]. ERK2DN expression resulted in lower levels of full-length cadherins (Fig. 5a). Detection with an anti-FLAG antibody was used to verify the expression of the construct, while actin detection was used to demonstrate equivalent protein loading.

Soft agar colony formation assays have been used to demonstrate the transformed feature and invasiveness of cultured cells [28]. Transfection of fibrosarcoma cells with the ERK2DN construct prior to plating for a colony formation assay resulted in an enhanced ability of the fibrosarcoma cells to form colonies in soft agar (Fig. 5b). Overexpression of ERK was theorized to be resulting in the inhibition of full-length cadherins and promotion of colony formation. Therefore, blocking ERK activity should restore control of colony formation. In support, treatment of fibrosarcoma cells with a MEK inhibitor (PD98059) caused a decrease in the ability to form colonies (Fig. 6), highlighting the necessity of the ERK pathway for invasiveness.

Discussion

The regulation of MAPK pathways upon attainment of contact inhibition has been previously studied in this laboratory. Normal human fibroblasts have been shown to have a decrease in the level of phosphorylated ERK [18] and p38 [19] upon contact inhibition, and changes in this MAPK regulation had downstream effects on the ability of normal fibroblasts to respond to oxidative stress upon the attainment of contact inhibition, while fibrosarcoma cells, lacking density-dependent growth control mechanisms, did not demonstrate density-dependent changes in the response to oxidative stress [29]. Because of these differences in MAPK regulation upon contact inhibition, it is likely that other cellular responses may be influenced by the attainment of a contact-inhibited state, including the ability of cells to carry out movement or attachment-free survival.

Matrix metalloproteinases (MMPs) are one group of proteins which are involved in the breakdown of extracellular matrix components, giving them roles in migration, proliferation, invasion, and metastasis [24, 30]. During the process of degrading the extracellular matrix, MMPs cleaves proteins that are bound to the cell membrane and have extracellular domains [31], including growth factor receptors, proteoglycans, and adhesion proteins such as cadherins and integrins. Regulation of MMPs is tightly controlled, with both control of transcription through response elements, including AP-1 [29, 32] and inhibition by tissue inhibitors of metalloproteinases (TIMPs) [33]. MMP-9 is one example of an MMP that is controlled by MAPK activity. In this work, inhibition of ERK led to a reduction in MMP-9 expression, while H₂O₂ treatment, which activates MAPK [25] enhanced MMP-9 expression (Fig. 2). In head and neck squamous cell carcinoma (HNSCC), iron was shown to regulate MMP-9 through an ERK1/2-dependant pathway [34]. Likewise, in endothelial cells, activation of the ERK cascade was shown to be necessary for induction of MMP-9 expression [35]. The level of MMP-9 was found to decrease in confluent cells (Fig. 1), consistent with less active ERK seen at confluency. MMP-9 expression is thought to be responsible for the cleavage of the 80-kDa extracellular domain of E-cadherin, resulting in disrupted function and expression of E-cadherin. Increased MMP-9 activity may result in decreased levels of active E-cadherin and lead to an increase in cell migration [36, 37]. To determine if culture confluence influenced the expression of cadherins, western blot analysis was performed to detect the cadherin levels in both subconfluent and confluent fibroblasts and fibrosarcoma cells. Though full-length cadherins were detected in both cell types under subconfluent or confluent conditions, an 80 kDa fragment was detected only in subconfluent cells (Fig. 3). This immunoreactive band could be a cleavage product of cadherins, and was also detected in the control A549 cell lysate. This work utilized a pancadherin antibody, so current work in the laboratory is to identify a specific cadherin which may be targeted in this pathway. Though a measurable decrease in full-length cadherins was not detected, it is possible that intracellular activity of MMPs [38], or other proteases, can be influenced by confluency, and could result in the detectable fragments observed here. Proteolytic cleavage of cadherins has been shown to be influential in cellular adhesion and intracellular signaling [39, 40]. Cleavage of E-cadherin by γ -secretase results in C-terminal fragment (E-cad/CTF2) which undergoes nuclear translocation and may regulate apoptosis [41]. Expression of a cadherin cytoplasmic domain in MDCK cells resulted in decreased cell surface localization of E-cadherin and increased migration [42]. Loss of cadherins decreases adhesion and may promote metastasis [43, 44].

To determine if modulation of ERK activity, as is seen upon contact inhibition [18], could be involved in the regulation of cadherin expression and invasiveness of cells, we sought to alter ERK activity through the use of the MEK inhibitor PD98059. Inhibition of MEK resulted in a potent decrease in both ERK activity and an increase in the expression of full-length cadherins (Fig. 4). There appeared to be no significant change in the amount of cleaved products seen. However, it should be noted that subconfluent cells were utilized for this assay, which had been displaying consistently high levels of an 80 kDa fragment. Conversely, excessive activity of ERK would be expected to decrease the expression full-length cadherins, and increase the invasive capacity of cells. Previous work has shown that contact-inhibited fibroblast cells have increased levels of MKP proteins [18]. In this work,

overexpression of an ERK2 construct with a mutation (D319N), making it resistant to phosphatase regulation resulted in both decreased levels of full-length cadherins and an enhanced ability to form colonies in soft agar (Fig. 5). Since MKPs are negatively regulated upon contact inhibition, these results suggest that MKP activity may play a role in the control of ERK-mediated cadherin degradation. Negatively regulating ERK through the use of PD98059 subsequently decreased the ability of fibrosarcoma cells to form colonies in soft agar (Fig. 6).

Taken together, these results corroborate a role for the regulation of ERK upon the attainment of a contact-inhibited state with increased expression of cadherins. Negative regulation of ERK, including dephosphorylation by MKPs, may play a role in allowing accumulation of full-length cadherins, while expression of MMPs in subconfluent cells may contribute to cadherin degradation and the promotion of migration. Current work in the laboratory is to further explore the direct influence of MMP-9 expression on cadherin activity. Further study will be undertaken to determine the potential influence of redox state regulation on cadherin expression and degradation, as previous work has shown that redox state changes occur during the transition of normal fibroblasts to a contact-inhibited state [45].

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Fig. 1.

Expression of MMP-9 decreases upon attainment of confluence in normal fibroblasts (BJ) and fibrosarcoma cells (HT-1080). (a) MMP-9 detection in subconfluent (S) and confluent (C) cultures. Results are representative of 10 independent experiments. The Coomassie stained membrane and actin detection are shown to indicate equal loading of protein. (b) Densitometry of the ratio of MMP-9 to actin expression is shown for in both normal fibroblasts (* $t < 0.03$) and fibrosarcoma cells(* $t < 0.04$).

Fig 2.

Expression of MMP-9 is influenced by ERK activity. Fibrosarcoma cells (HT-1080) were serum-starved overnight and subsequently treated with 50 μ M PD9859 for 24 hrs (a) or 300 μ M H_2O_2 for 10 min (b), and lysate was used to detect MMP-9 and actin. Results are representative of 3 independent experiments. The actin detection (a) or Coomassie stained membrane (b) are shown to indicate equal loading of protein.

Fig. 3.

Pan-cadherin expression in both subconfluent and confluent normal fibroblasts (BJ) and fibrosarcoma cells (HT-1080). The control contains A-431 cell lysate as a positive control for the antibody. Lysate from both subconfluent and confluent fibroblasts and fibrosarcoma cell cultures was used to detect cadherins. Results are representative of 9 independent experiments. The Coomassie stained membrane and actin detection are shown to indicate equal loading of protein.

Fig. 4.

Expression of phosphorylated ERK is decreased, and full-length cadherin is increased in subconfluent fibrosarcoma cells (HT-1080) treated with an inhibitor of MEK. Cells were serum-starved overnight and subsequently treated with 50 μ M PD9859 for 24 hrs. Treatment with the MEK inhibitor resulted in decreased expression of phosphorylated ERK and increased expression of full-length cadherins. Coomassie stained membranes and actin detection were used to demonstrate equal loading of proteins. Results shown are representative of 6 independent experiments.

Fig. 5.

Over-expression of constitutive ERK (ERKD319N) in fibrosarcoma cells (HT-1080) results in decreased expression of full-length cadherins and an enhanced ability to form colonies in soft agar. (a) Cells were transfected with pSR α -FLAG or pSR α -FLAG-ERK2DN and assayed for protein expression after 48 hrs. FLAG detection demonstrates expression of the construct, while actin detection was used to demonstrate equivalent loading of proteins. Results shown are representative of 3 independent experiments. (b) Cells transfected with either pSR α -FLAG or pSR α -FLAG-ERK2DN constructs were plated on soft agar 48 hrs. after transfection and were allowed to proliferate for 10 days. Colonies were stained with crystal violet and counted. Results are the average of 8 independent experiments, and were found to be statistically significant (* $p < 0.05$) using a t test.

Fig. 6.

Inhibition of MEK results in decreased ability of fibrosarcoma cells (HT-1080) to form colonies in soft agar. (a). Cells were seed onto soft agar plates and immediately treated with 50 μ M PD98059 (MEK inhibitor) or left untreated. Plates were re-fed every two – three days for 10 days, and then stained with crystal violet. (b). Average number of colonies per plate from treated (PD98059) and untreated control cells. Results are representative of four independent experiments and were found to be statistically significant (* $p < 0.01$) using a t test.