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Isoform-specific modulation of pressure-stimulated cancer cell proliferation and adhesion by alpha-actinin

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Abstract

Introduction—Intratumoral pressure may stimulate cancer proliferation while intravascular pressure promotes metastatic adhesion. α -actinin proteins facilitate focal adhesion formation and link focal adhesion complexes to the cytoskeleton. We hypothesized that α -actinin is the mechanotransducer that mediates the effects of pressure on cancer cell proliferation and adhesion.

Methods—We treated SW620 colon cancer cells with specific siRNA to reduce α -actinin-1 and/or α -actinin-4, the two key epithelial isoforms. Proliferation was measured in adherent cells by MTT assay after 24 hours at ambient or 40 mmHg increased pressure. For comparison, we evaluated the effects of 30 minutes of ambient or 15 mmHg increased pressure on adhesion of suspended SW620 cells. Because the transcription factor NF- κ B influences proliferation, we used co-immunoprecipitation to evaluate NF- κ B- α -actinin association and a lentiviral reporter assay for NF- κ B activity.

Results—40 mmHg increased pressure increased SW620 proliferation $41 \pm 6\%$ ($n=10$; $p<0.05$) vs. ambient pressure controls. Reducing α -actinin-1 and α -actinin-4 together or α -actinin-4 alone blocked this effect, but reducing α -actinin-1 alone did not ($n=6$; $p<0.05$). We observed a $72 \pm 11\%$ increase in NF- κ B activity ($n=6$; $p<0.05$), and increased association between NF- κ B and α -actinin-4 in adherent cells under pressure. NF- κ B and α -actinin-1 did not co-immunoprecipitate. However, reducing α -actinin-4 did not prevent pressure-induced NF- κ B activation ($n=8$).

Conclusions— α -actinin-4 may mediate pressure stimulation of proliferation within large rapidly growing tumors, perhaps by binding transcription factors such as NF κ B. α -actinins may be important targets to inhibit cancer proliferation and metastasis.

Introduction

Interstitial pressures in rapidly growing solid tumors are increased 4–50 mmHg above ambient conditions [1–2]. Animal models suggest that pressure increases even further as the tumor becomes more invasive [3]. Additionally, normal gut mucosa is subjected to pressure increases during normal peristalsis, and these pressures can increase significantly in diseased states as well as during and after medical interventions [4–5]. Many studies from our

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laboratory as well as others indicate that increased extracellular pressure is important in cancer cell signaling and metastatic progression.

Preliminary studies from our laboratories have shown that 15 mmHg increased extracellular pressure mediates proliferation in several colon cancer cell lines [6]. While the mechanism by which pressure stimulates proliferation has yet to be fully elucidated, preliminary evidence suggests it requires PKC but not Src or Akt.

The α -actinin family of proteins comprises a set of ubiquitously expressed actin-crosslinking proteins that interact with multiple focal adhesion proteins and are necessary for focal adhesion formation and turnover. α -actinin is also thought to provide a direct link between the actin cytoskeleton and integrins. We have previously reported that α -actinin-1, but not α -actinin-4, is critical in the early part of pathway that mediates the effects of pressure on cancer cell adhesion [7]. Therefore, we sought to test the hypothesis that α -actinin-1 may also be important in the mechanosensing involving pressure induced proliferation. Interestingly, and contrary to our initial hypothesis, we found that it is α -actinin-4, not α -actinin-1 that modulates pressure induced proliferation by a mechanism that may involve transcription factors such as NF- κ B.

Materials and Methods

Cell culture

We studied SW620 human colon cancer cells in Leibovitz L-15 media, and maintained pressure at 40 ± 1.5 mmHg for 24 hours using an airtight Lucite box with an inlet and an outlet valve for pressure application and manometer connection, as previously described [8].

Transfection

50nm of double stranded siRNA targeted the mRNA 5' - CACAGAUCGAGAACAUCGAAG-3' and 5' - CCACAUCAGCUGGAAGGAUGGUCUU-3' for α -actinin-4, α -actinin-1 respectively (Dharmacon, Lafayette, CO). Dharmacon siCONTROL non-targeting siRNA was a control. All studies were performed 48 hours after transfection.

Proliferation

5,000 cells/well were seeded in collagen I precoated 96-well plates and exposed to increased or ambient pressure for 24 hours before MTT assay per ATCC protocol.

Co-precipitation

After lysis in Tris buffer [8], protein was assayed by bicinchoninic acid (BCA) protocol (Pierce, Rockford, IL). For co-precipitation, 400 μ g protein was incubated with antibody for 1 hour, and then with agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. Equal protein amounts were resolved by SDS-PAGE and transferred to Hybond nitrocellulose (GE Healthcare, Buckinghamshire, UK) for Western blot. Mouse monoclonal anti- α -actinin-4 or rabbit polyclonal anti-NF- κ B were used for immunoprecipitation or Westerns.

NF- κ B activation

We assayed NF- κ B activity via a luciferase-based NF- κ B lentiviral reporter (Qiagen, Frederick, MD). 24 hours after plating 5,000 cells/well, lentiviral particles were introduced via SureENTRY reagent (2 μ g/mL, Qiagen) for 24 hours. The lentiviral suspension was replaced with normal medium, and cells were exposed to ambient or increased pressure for

24 hours. Bright-glow luciferin was added (Promega, Madison, WI) and quantitated by a FLUOstar Omega plate reader (BMG LabTech, Offenburg, Germany).

Statistics

Results are shown as $X \pm SE$ and analyzed via t-test, seeking 95% confidence.

Results

α -actinin-4, but not α -actinin-1, mediates SW620 cancer cell proliferation

We first sought to determine whether reducing α -actinin-1 influenced pressure-induced proliferation, as originally hypothesized. An MTT cell proliferation study following transfection with siNT, siActinin-1 or siActinin-4 indicated that pressure increased proliferation $41 \pm 6\%$ (Figure 1, $n=10$; $p<0.05$), relative to ambient pressure controls transfected with the non-targeting siRNA. Reducing both α -actinin-1 and 4 together or reducing α -actinin-4 alone blocked this effect, but reducing α -actinin-1 alone did not ($n=6$; $p<0.05$).

Increased extracellular pressure activates NF- κ B

The transcription factor NF- κ B may become activated during cellular stress and may mediate metastatic progression. We measured NF- κ B activation in response to increased extracellular pressure using a lentiviral NF- κ B reporter assay. 24 hours of increased pressure stimulated NF- κ B activity $72 \pm 11\%$ relative to ambient pressure controls (Figure 2, $n=6$; $p<0.05$).

α -actinin-1, but not 4, co-immunoprecipitates with NF- κ B

Co-immunoprecipitation from lysates of cell exposed to ambient or increased pressure showed a $36 \pm 5\%$ increase in NF- κ B/ α -actinin-4 association in pressure treated cells relative to ambient control (Figure 3). α -actinin-1 and NF- κ B did not display increased co-immunoprecipitation in response to elevated pressure.

Reduction of α -actinin-4 does not inhibit NF- κ B activation by pressure

To determine if pressure-induced NF- κ B activity requires α -actinin-4, we transfected the cells with α -actinin-4 specific siRNA or siNT RNA during the lentiviral reporter assay. After exposure to ambient or increased pressure, NF- κ B activity increased similarly in response to pressure in control cells as in the α -actinin-4 reduced cells ($65 \pm 8\%$ and $69 \pm 5\%$, respectively) (Figure 2, $n=7$; $p<0.05$).

Discussion

Intratumoral pressure is increased in diverse human and animal tumors, as the tumor grows rapidly against constraints [1–2]. This study suggests that tumor cells sense this increased extracellular pressure via α -actinin-4, initiating intracellular signals that stimulate proliferation. This may initiate a vicious positive feedback loop in which tumor growth stimulates pressure which in turn stimulates more rapid growth. NF- κ B is also activated within tumor cells by pressure. NF- κ B associates with α -actinin-1 and α -actinin-4, but increases in its association only with α -actinin-4 in response to pressure. Since NF- κ B influences both proliferation and apoptosis in a complex fashion, NF- κ B may also contribute to the mitogenic effects of pressure.

α -actinin-4 is an actin crosslinking protein that regulates focal adhesions in other cell types under ambient pressure. The α -actinin protein family interacts with diverse cytoskeletal and

signaling proteins, including paxillin in colon cancer cell lines [8], MAPK in NIH3T3 fibroblasts [9], and PKN in cos-7 cells [10]. α -actinin-4 mutations are associated with decreased podocyte adhesion [11]. Although others have associated α -actinin-4 with invasion and metastasis in colorectal cancer [12], this is the first demonstration that α -actinin-4 contributes to the regulation of proliferation by physiological increases in extracellular pressure. α -actinin-4 seems critical to the pathway by which cancer cells respond to physical stress, and increases their proliferative response.

We have previously described a complex pathway by which even a transient 15 mmHg increase in pressure modulates adhesion to matrix proteins, endothelial cells, and surgical wounds in diverse cancer cell lines and primary cancer cells [13–14]. This requires α -actinin-1. Downstream, FAK, Src and Akt activation lead to increased phosphorylation and binding affinity of β 1-integrin [14]. Pressure stimulation of cancer cell proliferation is mediated differently than pressure effects on cancer cell adhesion [6]. Src or Akt inhibition did not prevent pressure-mediated proliferation in our previous work. We now show that even at the mechanosensing upstream end of the pathway α -actinin-4 but not α -actinin-1 is required for pressure to stimulate proliferation in contrast to the signals by which pressure promotes adhesion. Pressure stimulates integrin binding affinity and adhesiveness in cells suspended in fluids such as medium, lymph, blood, or wound irrigation fluid, while pressure stimulates proliferation in cancer cells attached to the extracellular matrix. Differences in the cytoarchitecture of suspended and adherent cells may therefore explain the different roles of α -actinin-4 and α -actinin-1 in these two settings.

NF- κ B is a rapidly acting transcription factor that mediates transcription in cell cycle regulation, apoptosis and proliferation [15]. NF- κ B influences cancer development and progression, but the implications of NF- κ B activation are sometimes conflicting. For instance, NF- κ B promotes apoptosis in colon cancer cells treated with aspirin, but constitutive NF- κ B activation promotes gastric cancer cell proliferation [16–17]. Our data show that increased extracellular pressure increases NF- κ B activity and NF- κ B association with α -actinin-4. Others have shown nuclear co-localization of α -actinin-4 with NF- κ B in human epidermoid carcinoma cells during EGF activation by co-immunostaining, but the NF- κ B- α -actinin-4 interaction upon cellular stress was not studied [18]. Other transcription factors, including Rac1 and AP-1, are also activated under cellular stress [8, 19]. Whether these factors also contribute to the pathway by which pressure modulates proliferation awaits further study.

In sum, α -actinin-4, but not α -actinin-1, critically mediates the stimulation of cancer cell proliferation by increased extracellular pressure. This pathophysiological pressure increase activates NF- κ B and causes α -actinin-4-NF- κ B association, although this association appears unnecessary for NF- κ B activation. How α -actinin-4 and NF- κ B interact awaits further study, but α -actinin-4 may be a therapeutic target, especially for patients with unresectable solid tumors in whom it may be desirable to inhibit pressure-mediated mitogenic stimuli.

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Figure 1. -actinin-4, but not -actinin-1 is necessary for pressure induced proliferation
 SW620 human colon cancer cells were transfected and exposed to ambient (open bars) or 40 mmHg increased pressure (shaded bars) for 24 hours. Pressure treated cells displayed significantly increased proliferation. This effect was abolished in cells that were transfected with small interfering RNA specific for -actinin-4 and -actinin-1 in combination, as well as -actinin-4 alone. Transfection with -actinin-1 small interfering RNA alone had no effect.

Figure 2. NF- κ B activity increases in response to increased extracellular pressure independent of α -actinin-4

40 mmHg increased extracellular pressure (shaded bars) for 24 hours increased NF- κ B activity relative to ambient control cells (open bars). Treatment with α -actinin-4 specific siRNA had no effect on NF- κ B activity in response to increased pressure.

Figure 3. NF-kB co-immunoprecipitates with α -actinin-4, but not α -actinin-1

Equal protein samples from SW620 cells exposed to ambient or 40 mmHg increased pressure for 24 hours were immunoprecipitated for NF-kB and probed for α -actinin-4 and α -actinin-1 by western blotting. NF-kB and α -actinin-4 displayed increased western blotting in response to increased pressure, while NF-kB and α -actinin-1 did not.