PARP-1 modulates β1-integrin/NF-κB-mediated radioresistance in human breast cancer

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Abstract
Genomic damage triggers a signal transduction network that modulates gene expression and protein stability to repair DNA and influence cell survival. Inhibition of the DNA damage response protein PARP-1 is the subject of ongoing preclinical and clinical investigations in breast cancer, where DNA damage activates protective signaling networks that are transmitted through the extracellular matrix by the transmembrane protein β1-integrin. β1-integrin influences gene expression by modulating activity of the transcription factor NF-κB, yet the mechanism by which DNA damage activates β1-integrin is unknown. Here we demonstrate that breast cancer cells are enriched in PARP-1, and that PARP-1 activity is required for signaling through the tumor microenvironment in response to ionizing radiation. Moreover, PARP-1 and β1-integrin cooperatively regulate viability and growth of breast cancer cells propagated in three dimensional laminin-rich extracellular matrix (3D lrECM) cultures. Finally, we show that PARP-1 interacts with both β1-integrin and NF-κB in response to genotoxic stress to link signaling through the extracellular matrix to changes in gene expression. The data are consistent with a model whereby PARP-1 inhibition sensitizes cancer cells to the cytotoxic effects of DNA damage by coordinated disruption of both tumor microenvironment- and NF-κB-signaling pathways.

Keywords: Breast cancer, tumor microenvironment, PARP-1, β1-integrin, NF-κB

Introduction
Tumor cell interaction with the extracellular matrix is a significant source of acquired or developed cancer therapy resistance in human cancers [1,2]. These signals are transduced by integrins, a large family of transmembrane glycoproteins that are phosphorylated to regulate diverse cellular processes [3]. In breast carcinoma, β1-integrins are aberrantly expressed to influence cell fate, organization, survival, apoptosis, and acquired resistance to human epidermal growth factor inhibitor [4-6]. Indeed, β1-integrin expression is predictive for both death and development of metastatic disease in human breast cancer patients, and has therefore garnered considerable interest as a target for molecular therapeutics in recent years [7,8].

We recently demonstrated that β1-integrin promotes both invasiveness and radioresistance through cooperative signaling with the ubiquitous transcription factor nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) [9,10]. Mammalian NF-κB is a dimer composed of varying subunits depending on the cellular context, including p50, p52, p65 (RelA), c-Rel, and RelB. Activation of NF-κB signaling occurs upon degradation of inhibitor-κB (IκB) proteins, which allows NF-κB to translocate from the cytoplasm to the nucleus and bind specific κB-sites to regulate gene expression (www.NF-κB.org) [1,11].

The DNA damage response (DDR) is an evolutionarily conserved signal transduction network that has been implicated in NF-κB activation [12-15]. In BRCA-deficient breast cancer, small molecular inhibitors of the DNA-dependent nuclear enzyme poly(ADP-ribose) polymerase (PARP) lead to synthetic lethality and are the subject of ongoing preclinical and clinical investigation as radiosensitizers and adjuvant therapies [7,16,17]. PARP-1 plays a pivotal role in the DDR, and also modulates angiogenesis, metastasis, metabolism, survival, chromatin structure, and NF-κB-mediated tumor inflammation [9,10,18,19]. In particular, PARP-1 interacts with NF-κB independent of DNA binding or PARP-1 enzymatic activity to regulate signaling through the TME by activating fibronectin (FN1) and intercellular adhesion molecule 1 (ICAM-1) transcription [20-23]. Moreover, PARP-1 mediates radioresistance in response to ion-
izing radiation-induced NF-κB activation without influencing IκBα degradation or nuclear translocation of p50 or p65 [24].

Given the parallels between the PARP-1, NF-κB and β1-integrin activity in response to DNA damage, we hypothesized that PARP-1 connects TME and NF-κB signaling pathways to regulate cellular radioresistance. The data demonstrate that PARP-1 interacts with both β1-integrin and NF-κB, and that PARP-1 inhibition attenuates TME- and NF-κB-signaling to sensitize tumor cells to the cytotoxic effects of ionizing radiation. The heretofore-unidentified mechanist connection between PARP-1 and β1-integrin signaling in breast cancer suggests that PARP-1 inhibition disrupts both TME and NF-κB signaling to sensitize human malignancies to DNA damage.

Materials and methods

Cell culture

Non-malignant S1 and malignant T4-2 human breast cancer cells were maintained as described previously [25]. For experimentation, S1 and T4-2 cells in complete media were seeded as single cells in three-dimensional (3D) laminin-rich extracellular matrix (lrECM) (Matrigel, Trevigen, MD). For experiments involving the PARP inhibitor AG-14361 (Selleckchem, Houston, TX), drug or vehicle control was added after 4 or 6 days for T4-2 and S1 cells, respectively (Figure 1A). After 24 hours of exposure, cells were radiated and collected for analysis at the indicated time points [26,27].

Electrophoretic mobility shift assay (EMSA)

Cells in 3D lrECM cultures were harvested using the nuclear extraction kit (Thermo Scientific, Rockford, IL) following the manufacturer’s specifications. Protein concentration was determined using the DC Protein Quantitation Kit (Bio-Rad, Hercules, CA), and NF-κB DNA binding activity was quantified using the TransAM™ NF-κB TF assay kit (Active Motif, Carlsbad, CA). In brief, 10 μg of nuclear extract in cell binding and cell lysis buffer were added in each well in streptavidin-coated 96-microtiter plates in triplicate. Nuclear extract from the Burkitt’s lymphoma Raji cell line was used a positive control. Oligonucleotides containing a 10 bp NF-κB binding motif (wild type or mutated) from the human β1-integrin promoter were custom synthesized and biotinylated (Midland Certified Reagent Company, Midland, TX). Excess wild-type NF-κB consensus oligonucleotide (20 pmol/well) or an inactive mutated consensus oligonucleotide was added to nuclear extracts. After washing, wells were sequentially incubated with a primary antibody against p65 NF-κB (Cell Signaling), followed by anti-rabbit peroxidase-conjugated antibody (GE Health Care, United Kingdom). After substrate addition, peroxidase activity was measured by reading 450 nm in a SpectraMax® Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA).
**Immunoblotting**

Cells grown in 3DlrECM were extracted using ice-cold PBS (pH 7.2) containing 5 mM EDTA, and lysed in radioimmunoprecipitation assay (RIPA) buffer as described previously [25]. After protein quantification using the DC Protein Quantitation Kit (Bio-Rad), equal amounts of protein were loaded onto sodium dodecyl sulfate gels (Invitrogen, Carlsbad, CA). After polyacrylamide gel electrophoresis, samples were transferred onto PVDF membrane (Millipore, Temecula, CA) and blocked with 5% nonfat milk before incubation with antibodies. Lastly, blots were visualized using the ECL Western blotting detection system (Thermo Scientific). The following primary antibodies were used: PARP-1 (Calbiochem, CA); β1-integrin, clone 18 (BD Transduction Laboratories, Lexington, KY); phospho-β1-integrin (Biosource International, Camarillo, CA); FAK (BD Transduction, San Jose, CA); pFAK (BD Transduction); cleaved caspase 3 (Cell Signaling, Beverly, MA); and β-actin (Sigma, St. Louis, MO). Densitometry was performed using Image J software, and expression was normalized against either β-actin or unphosphorylated cognate protein.

**Immunoprecipitation**

Protein was extracted using lysis buffer at 4°C containing 1% Brij 98, 150 mM NaCl, 25 mM HEPES, 5 mM MgCl2 and 1% eukaryotic proteinase inhibitor cocktail (Calbiochem). Extracts were pre-cleared for 1 hr with normal rat immunoglobulin G and 25 µl of Dynabeads® protein G (Invitrogen) at 4°C, and then incubated overnight at 4°C with anti-PARP or anti-β1-integrin antibodies (Argon Bioscience, Morgan Hill, CA). Immunoprecipitates were captured by an additional 1 hour incubation with 25 µl of Dynabeads® protein G. Lastly, beads were collected, washed, and prepared for analysis on 4-20% Novex® Tris-Glycine gels (Invitrogen).

**Proliferation and apoptosis assays**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was used for apoptosis detection [28]. In brief, T4-2 3D IrECM cell cultures were collected on day 7, fixed onto glass slides in 4% paraformaldehyde, and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. Samples were then washing and incubated in TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche) at 37°C for 1 hr. Cells were counterstained with 4',6-diamino-2-phenylindole (DAPI) at room temperature for 5 min, washed and mounted. Fluorescent images were captured using a ZEISS microscope equipped with an AxioCam HRm camera.

Ki-67 nuclear antigen (Novocastra Laboratories, Norwell, MA) was used to stain cells for immunofluorescence. In brief, samples were fixed in methanol/acetic acid, blocked using 10% goat serum in IF buffer (0.05% NaN3, 0.1% BSA, 0.2% Triton-X 100 and 0.05% Tween 20 in PBS) and treated with 1:250 dilution of goat anti-mouse IgG Fab fragments (Life Technology, Carlsbad, CA). Samples were incubated overnight at 4°C with Ki-67 antibodies, washed, and then treated with FITC-conjugated anti-rabbit secondary antibodies (Jackson Laboratory, Bar Harbor, ME). Nuclei were counterstained with DAPI, and images were again captured using a ZEISS microscope equipped with an AxioCam HRm camera.

**RNA isolation and quantitative real-time PCR (qRT-PCR)**

3D lrECM cell cultures were extracted as described above, and RNA was purified using the Qiagen RNeasy kit according to the manufacturer’s instructions (Qiagen, Venlo, Limburg). The M-MLVkit was used for reverse transcription of 1.5 µg of each sample (Invitrogen). qRT-PCR was carried out using 2 µl of cDNA from each sample with the SYBR® Green PCR Kit (Invitrogen). Samples were amplified and analyzed using Step One Software v2.3 (Applied Biosystems, Carlsbad, CA) at 95°C for 10 min, followed by 40 amplification cycles of 94°C for 15 s followed by 60°C for 60 s. Primer sequences are as follow; β1-integrin (forward: 5’-AGGTGGTTTGCAGCCTCAT-3’, 5’-AAGTGAAACCCGGCATCTGTG-3’), and β-actin (Sigma, St. Louis, MO). Densitometry was performed using Image J software, and expression was normalized against either β-actin or unphosphorylated cognate protein.

**Statistics**

Unpaired, two-tailed Student’s t-test were used to compare groups, and statistical significance was defined as P<0.05 (*). Unless specified otherwise, data are displayed as the average value of at least 3 biologic replicates±standard deviation, and are representative of at least 2 independent experiments.

**Results**

We have previously shown that NF-κB and β1-integrin cooperate to enhance radioresistance in breast cancer cell culture through a feed forward mechanism [9]. We therefore sought to determine if DDR effectors orchestrate β1-integrin/NF-κB signaling to protect tumor cells from DNA damage-induced cytotoxicity. To do so, T4-2 breast cancer cells were grown in 3D IrECM, which better mimics physiologic growth conditions and interactions with the TME than 2D culture. When grown in 3D conditions, T4-2 cell cultures are enriched in PARP-1 expression compared to nonmalignant S1 acinar structures ([Figure 1B]). Moreover, PARP-1 expression is intensified in T4-2 3D lrECM cell cultures as compared to nonmalignant S1 acinar structures ([Figure 1B]). Consistently, PARP-1 expression is intensified in T4-2 3D lrECM cell cultures as compared to nonmalignant S1 acinar structures ([Figure 1B]). Consistently, PARP-1 expression is intensified in T4-2 3D lrECM cell cultures as compared to nonmalignant S1 acinar structures ([Figure 1B]). Consistently, PARP-1 expression is intensified in T4-2 3D lrECM cell cultures as compared to nonmalignant S1 acinar structures ([Figure 1B]). Consistently, PARP-1 expression is intensified in T4-2 3D lrECM cell cultures as compared to nonmalignant S1 acinar structures ([Figure 1B]).
PARP-1 inhibition attenuates the expression and irradiation-induced phosphorylation of focal adhesion kinase (FAK), a cytosolic tyrosine kinase involved in the transduction of diverse signals in response to integrin engagement (Figure 2B, Supplementary Figure 2B) [32]. qRT-PCR assessment of transcript levels suggests that these findings are not the result of changes in protein synthesis, but rather the product of altered protein stability. In this regard, ionizing radiation fails to alter β1-integrin mRNA expression in either S1 or T4-2 colonies (Figure 2C).

Given the importance of PARP-1 activity for integrin-mediated signaling, we next investigated the effect of PARP-1 inhibition on breast cancer organoid growth and proliferation. T4-2 3D lrECM colonies were treated with AG14361 or vehicle control with or without irradiation, and imaged (Figure 3A). Both PARP-1 blockade and the β1-integrin inhibitory antibody ABII2 significantly reduced acinar diameter (Figure 3B). The addition of ionizing radiation further reduced organoid growth, although the magnitude of effect was blunted relative to either PARP-1 or β1-integrin inhibition alone. Similarly, PARP-1 and β1-integrin inhibition increased apoptosis (Figure 3C) and reduced cell proliferation (Figure 3D), the effect of each was synergistically amplified with ionizing radiation (Figure 3C).

Consistent with these data, PARP-1 inhibition increased the level of cleaved caspase-3 both at baseline and in response to irradiation (Figure 3E). Together, these data demonstrate that coordinated PARP-1/β1-integrin signaling attenuates apoptosis in response to DNA damage to regulate tumor cell proliferation and radioresistance.

To determine if PARP-1 acts directly on β1-integrin to regulate cell viability in response to DNA damage, reciprocal immunoprecipitation experiments were performed from T4-2 and S1 3D lrECM cell cultures. These experiments show that β1-integrin interaction with PARP-1 significantly increases in response ionizing radiation (Figure 4A). However, following PARP-1 inhibition with AG14361, β1-integrin recovery is comparable to background in both S1 and T4-2 cell lysates. Consistently, PARP-1 recovery from T4-2 anti-β1-integrin immunoprecipitates is radiation-dependent, and attenuates in the presence of AG14361 (Figure 4B). In combination with quantitative immunoblots, cell proliferation studies, and apoptosis assays, these protein binding data demonstrate that interaction with PARP-1 regulates β1-integrin expression and activity to modulate radioresistance following DNA damage.

NF-κB p65 regulates invasiveness and radioresistance of breast cancer cells in response to β1-integrin signaling [9,33].
Figure 3. PARP-1- and β1-integrin-signaling attenuate apoptosis to regulate tumor cell proliferation and radioresistance.

(A) Morphology, TUNEL and Ki-67 staining of T4-2 3D lrECM cell culture with or without AG14361 and/or irradiation.
(B) Morphometric quantification of T4-2 acinar diameter demonstrates that PARP-1 and β1-integrin inhibition impairs cell growth.
(C) TUNEL assay shows increased T4-2 cell death when either PARP-1 or β1-integrin inhibition is combined with radiation.
(D) Ki-67 staining confirms that T4-2 proliferation is lowest when either PARP-1 or β1-integrin inhibition is combined with radiation.
(E) Quantitative immunoblots demonstrate that expression of cleaved caspase 3 is increased with PARP-1 inhibition alone, and further increases with the addition of radiation.

In this pathway, NF-κB also binds to the promoter region of β1-integrin to modulate integrin expression and extracellular matrix signaling through a feed forward mechanism. Given that PARP-1 was found to influence β1-integrin signaling in response to DNA damage, we sought to determine if PARP-1 also modulates NF-κB activity. EMSA with oligonucleotides containing wild type or mutated κB binding sites was therefore performed from T4-2 3D lrECM nuclear extracts in the presence or absence of AG14361. These experiments demonstrate that PARP-1 inhibition attenuates radiation-induced p65 DNA binding (Figure 5A). This effect appears to be mediated through interaction between PARP-1 and NF-κB, as p65 is enriched in T4-2 3D lrECM anti-PARP-1 immunoprecipitates following ionizing radiation, but diminished upon addition of AG14361 (Figure 5B).

In sum, the data support a model whereby PARP-1 links β1-integrin signaling in response to DNA damage to the NF-κB transcriptional program to modulate the cellular response to ionizing radiation.

Discussion

PARP-1 regulates NF-κB activation by multiple mechanisms, which vary not only according to PAR acceptor protein, but also NF-κB subunit identity and signalosome composition [19]. Here we demonstrate that PARP-1 regulates both TME- and NF-κB signaling in response to DNA damage to promote tumor cell survival in a 3D in vitro model of breast...
cancer. In this model, targeted agents against either PARP-1 or β1-integrin promote apoptosis and impair tumor cell survival and growth after ionizing radiation. These effects appear to be mediated by interaction between PARP-1 and β1-integrin, and also between PARP-1 and NF-κB. It remains to be established if β1-integrin, either in its full-length form or after proteolytic processing, also interacts with NF-κB to modulate transcriptional behavior in response to ionizing radiation. Moreover, it is unclear if such an interaction would be a relevant therapeutic target in combination with PARP-1 inhibition. The majority of combination molecular therapies have proven to be overly toxic in most human trials to date, and ongoing investigation is required to identify new means of achieving synthetic lethality. In the interim, the data presented here warrant investigation of combined PARP-1 and β1-integrin antagonism within \textit{in vivo} breast cancer models.

Consistent with the observation that many cancers display misregulation of the DDR, PARP-1 protein expression is elevated in breast cancer cells relative to wild type mammary epithelia both at baseline and in response to DNA damage \cite{16}. Although specific DDR defects have not been identified for the majority of malignancies, the data presented here illustrate that PARP-1 exerts a pro-survival effect in breast cancer that is independent from its function as a DNA repair enzyme \cite{34}. Thus, traditional assays for evaluating the fidelity of DNA repair may overlook many non-canonical effects of mutated or misregulated DDR proteins in human malignancies. Indeed, evolving understanding of the molecular connections between the DDR and other pro-survival cellular pathways in human cancers is likely to identify additional targets for therapeutic intervention.

Our data demonstrate that PARP-1 connects the DDR and NF-κB-mediated transcription to pro-survival signaling through the TME. The role of NF-κB in the DDR is complex, and both the activation and behavior of NF-κB appear to depend on the type of genomic lesion and cellular context in which the damage occurs \cite{13}. We previously reported that radiation-induced β1-integrin expression in human breast cancer cells was mediated by NF-κB \cite{9}. In support a functional connection between TME, NF-κB, and DDR signaling, we observed an increase in \textit{β1-integrin} transcript levels following PARP-1 inhibition in breast cancer cells. This observation is consistent with a compensatory mechanism to maintain a homeostatic signaling balance between the TME and DDR in malignancy, but it is notable that a similar response was not seen in non-malignant cell culture. The data presented here do not establish a definitive connection between heightened TME signaling and malignancy, but the discrepancy in \textit{β1-integrin} transcription following PARP-1 inhibition is certainly consistent with such a model.

\textbf{Conclusion}

Our data demonstrate that a functional link exists between
PARP-1 activity in response to DNA damage, pro-growth signals through the TME, and NF-kB-mediated transcription in human breast cancer cell culture. These datashot light onto the process by which PARP-1 inhibition sensitizes breast cancer cells to genotoxic therapies, and serve as a rational for pre-clinical studies of combination PARP-1 and β1-integrin inhibitors within in vivo breast cancer models.

Additional files

Supplementary Figure S1
Supplementary Figure S2

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

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