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Priming and potentiation of DNA damage response by fibronectin in human colon cancer cells and tumor-derived myofibroblasts

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Abstract

We have previously shown that the genotoxin-induced apoptosis in mouse embryo fibroblasts was enhanced by the extracellular matrix protein fibronectin (FN). In the present study, we tested the hypothesis that FN regulates the DNA damage response (DDR) signaling pathways in HCT116 (p53-wt) and HT29 (p53-mut) human colon cancer cells and tumor-derived myofibroblasts. DNA damage recognition mechanisms were analyzed by immunofluorescence staining, cell cycle analysis and immunoblotting addressed at specific molecular sensors and executors involved in the DDR pathways. The results show that FN, but not collagen type IV or Matrigel, initiates and potentiates the DDR to the anticancer drug cisplatin in an $\alpha 5$ integrin and cell cycle-dependent manner (S and G2/M phases) in human colon cancer cells. Accordingly, we demonstrate that adhesion of HCT116 cells to FN upregulated the expression of $\alpha 5$ integrin FN receptors at the cell surface. These FN-induced DDR pathways include the concerted phosphorylation of histone H2AX on Ser¹³⁹ detected as nuclear foci (γ -H2AX, 15 and 25 kDa forms), of ataxia telangiectasia mutated (ATM-Ser¹⁹⁸¹), checkpoint kinase 2 (CHK2-Thr⁶⁸, 62 and 67 kDa) and p53-Ser¹⁵. These FN-induced γ -H2AX signals were interrupted or attenuated by selective inhibitors acting on the DDR pathway kinases, including wortmannin (targeting the phosphatidylinositol-3-kinase-related protein kinases, PIKK), KU55933 (ATM), NU7026 (DNA-dependent protein kinase catalytic

subunit, DNA-PK-cs) and SP600125 (JNK2, stress activated protein kinase/c-Jun N-terminal kinase-2). Adhesion to FN also potentiated the γ -H2AX signals and the cytotoxic effects of cisplatin in human colon tumor-derived myofibroblasts. These data provide evidence that FN promotes DNA damage recognition and chemosensitization to cisplatin via the potentiation of the DNA damage signaling responses in human colon cancer cells and tumor-derived myofibroblasts.

Keywords

cisplatin; γ -H2AX; phosphatidylinositol-3-kinase-related protein kinases; ataxia telangiectasia mutated; check-point kinase 2; c-Jun-NH2-terminal kinase-2; p53; α 5-integrins; cell cycle; human colon tumor myofibroblasts

Introduction

Tumor malignancy depends upon a complex dialogue between multiple cell types operating within a dynamic extracellular matrix (ECM) ecosystem. The ECM is more than a mechanical barrier as it also serves as a passive and active substrate for cancer cells and tumor-associated stromal cells, such as myofibroblasts. The malignant phenotype of cancer cells is also regulated by complex cell-matrix interactions and the remodeling of the ECM to create growth and survival responses linked to tumor invasion and neoangiogenesis. Some studies show that collagens, laminin, fibronectin (FN), SPARC, and tenascins have been reported to be associated with poor prognosis in patients with colon cancer (1-4). Cell adhesion to the ECM is primarily mediated by the integrin receptor family of α and β subunits, which pair to form 24 different integrin heterodimers. Each heterodimer binds to specific ECM elements and can transmit distinct signals (3,5). The α subunit typically confers specificity for the ligand, whereas the β subunit couples to downstream signaling pathways (6). FN, a major component of the ECM, binds to several integrins, including α 5 β 1. Cell adhesion to FN is required for genotoxic drugs to activate the proapoptotic functions of p53 and nuclear Abl tyrosine kinase in fibroblasts and melanoma (7-9). Several agents including reactive oxygen species, ionizing radiation, carcinogens, and anticancer agents inducing replication and transcription stress initiate the DNA damage and repair pathways (10). DNA damage activates members of the phosphoinositide-3 kinase-like kinase (PIKK) family of protein kinases, ATM (ataxia telangiectasia mutated), the ATM- and Rad3-related protein kinase (ATR), and the DNA-dependent protein kinase (DNA-PK) to phosphorylate a host of proteins (11,12). Among these is H2AX, a nucleosomal histone variant present in ~10% of the nucleosomes, an important nuclear marker for PIKK activation (13). In DDR pathways, phosphorylated H2AX is known to detect DNA-double strand breaks (10). In response to DNA damage, activated PIKK rapidly phosphorylates H2AX at a conserved C-terminal Serine-Glutamine (SQ) motif in its carboxyl-terminus (amino acid residues 139 and 140) to facilitate DNA repair by maintaining repair and signaling factors in proximity to the DNA damage site (14). The Ser139-phosphorylated H2AX, termed γ -H2AX is a very sensitive molecular sensor of the DDR pathways (15,16). A recent study reported that the activator of DNA repair response γ -H2AX is essential for the proliferation of endothelial cells under hypoxia (17).

The aim of the current study was to test the hypothesis that adhesion of epithelial colon cancer cells and tumor-associated myofibroblasts to FN influence DNA damage responses (DDR) and γ -H2AX signals. We therefore investigated whether the differential DDR capacity of cells attached to FN are modified after exposure to the genotoxin cisplatin. To address these issues, we have employed a series of imaging investigations combined with cell cycle and molecular signaling analyses.

Materials and methods

Reagents

FN was purchased from Sigma-Aldrich (St. Louis, MO), Matrigel from BD Bioscience (San Jose, CA), and human collagen type IV from Chemicon International (Temecula, CA). Wortmannin was from Calbiochem (San Diego, CA), the ATM inhibitor KU55933 and the DNA-PK inhibitor NU7026 (18,19) were respectively from Dr Graeme C. Smith, KuDOS Pharmaceuticals (Cambridge, UK), and Professor Roger J. Griffin (University of Newcastle, Newcastle upon Tyne, UK). The JNK inhibitor SP600125 and cisplatin were respectively from Invitrogen (Cergy Pontoise, France) and Merck Pharmaceutical Company (Rodleben, Germany). Inhibitors and control vehicles were added to cell cultures so that the final DMSO concentration was kept constant at 0.25% (v/v).

Cell culture and MTT test

Human colon cancer cell lines HCT116 and HT29 (integrin α 5 deficient and proficient) were provided by Dr C. Richard Boland and Dr Katharina M. Detjen, respectively (20,21). Cancer cells were maintained at 37°C in 5% CO₂ and DMEM (Dulbecco's modified Eagle's medium, Invitrogen) containing 10% (v/v) fetal bovine serum (FBS, PAA Laboratory, Pasching, Austria), 200 mM L-glutamine, 100 mM sodium pyruvate and antibiotics (Invitrogen). HT29 cells expressing integrin α 5 were cultured in the presence of 0.4-0.8 μ g/ml G418 (Invitrogen) to ensure retention of α 5 subunit ectopic expression. Human colon-derived primary myofibroblasts were obtained from a patient with adeno-carcinoma submitted to surgical resection for therapeutic purposes (22). Myofibroblasts were isolated using explant culture. Briefly, tissue fragments were cut in pieces (2-3 mm³) and transferred into a 6-well plate with 100 μ l of FBS supplemented with antibiotics added on top of each fragment. Cultures were placed in a humidified 37°C, 10% CO₂ incubator for 24 h, followed by addition of 3 ml DMEM containing 10% FBS. After 3-6 days outgrowth was observed, colonies of primary cells were subcultured for 15 days. Myofibroblast cultures were vimentin and α -smooth muscle actin-positive and cytokeratin-negative. Myofibroblasts (2×10^4 cells per well) were seeded on BSA or FN coated 96-well plates for 24 h and then treated for 48 h with cisplatin (10 μ M). Cultured cells were subjected to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All assays were performed in triplicates for each condition.

Immunofluorescence staining

At specified times, medium was aspirated and cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Cells were washed twice in PBS and incubated for 1 h at 37°C or overnight at 4°C with primary antibodies in PBS, 3% FBS, and 1% Triton X-100, then

washed, incubated for 1 h at room temperature with Texas Red or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:1000) and DAPI (1 mg/ml). Images were captured with the DeltaVision restoration microscope system (Applied Precision Inc., Issaquah, WA) using a photometrics Sony Coolsnap HQ charged-coupled device camera system (10 MHz, 12 bit, 1392×1040) attached to an inverted, wide-field fluorescent microscope (Nikon TE-200). Images were analyzed on Silicon Graphics workstations using the DeltaVision software package (Softworx, Version 2.50). For each treatment antigen expression was determined in at least 50 cells with one of the following primary mouse monoclonal antibodies (mAb): anti-Ser-139 phospho-H2AX (γ -H2AX, Millipore-Upstate, 1:1000), anti-Ser-1981 phospho-ATM (Millipore-Upstate, 1:500), and anti Ser-15 phospho-p53 (Cell Signaling Technology, Beverly, MA, 1:500).

Western blots

Whole cell extracts were prepared in RIPA buffer (50 mM Tris, pH 7.5) containing 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1% NP-40, 0.5% sodium doxycholate, 0.1% SDS and supplemented with a cocktail of protease inhibitors (Boehringer Mannheim Corp., Indianapolis, IN). Proteins (50 μ g) were analyzed by pre-cast 4-10% polyacrylamide NuPAGE[®]Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membranes. Membranes were incubated for 1 h at room temperature with the γ -H2AX mAb (1:1000), rabbit pAb Thr⁶⁸ phospho-CHK2 (1:1000, Cell Signaling Technology), mAb Ser-15 phospho-p53 (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and mAb β -actin (1:20,000, Sigma-Aldrich, Chemie GmbH, Switzerland). Probed membranes were then rinsed and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Immunoreactive proteins were revealed using the ECI Advanced system (GE Healthcare, Aulnay Sous Bois, France).

Cell cycle analysis, γ -H2AX and integrin labeling

Cell cycle progression was determined by flow cytometry as described (23). The cell cycle profile was determined from the DNA fluorescence data using the Multicycle software (Phoenix Flow System Inc., San Diego, CA). The DNA content was expressed as DNA index (DI). Biparametric analysis of γ -H2AX labeling along with cell cycle progression was determined by flow cytometry. Cells were fixed, permeabilized and stained as described (24). Under these conditions, γ -H2AX is fully accessible for labeling. Analyses and sorting were performed with a Coulter EPICS XL flow cytometer (Beckman-Coulter, France). To assess the expression of cell surface FN-binding α 5 and β 1 integrins by flow cytometry (FACSCalibur[™] system, Becton-Dickinson, NJ), 10⁶ cells were suspended in 100 μ l PBS 3% BSA containing 20 μ g/ml of α 5 MAB1969 (1:50), β 1 MAB1997 (1:50), and control IgG (1:100) purchased from Millipore (Temecula, CA). Analysis was done using the WinMDI or CellQuest[™] programs (Becton-Dickinson).

Statistical analysis

Results are expressed as the mean \pm SEM, using the Student's t-test. P<0.05 was considered statistically significant.

Results

Promotion of H2AX phosphorylation by fibronectin

In asynchronous cultures of HCT116 colon cancer cells plated on BSA, heterogeneous distribution of nuclear γ -H2AX staining was observed in a subpopulation of control cells representing $3.5 \pm 1\%$ of the total cell population. Approximately 20% of γ -H2AX-positive cells plated on BSA contained small γ -H2AX foci (Fig. 1A, panels a-a') and 3% contained larger foci (panels b-b'). In cultures plated on fibronectin (10 $\mu\text{g/ml}$) and examined 24 h later, a significant increase in γ -H2AX signals was observed (panels c-c' and d-d'), showing γ -H2AX nuclear labeling in BSA and FN-coated HCT116 cells, respectively. Next, we explored the effect of two other extracellular matrix (ECM) components on γ -H2AX nuclear foci formation (Fig. 1A, panels e-h). We compared the γ -H2AX signals induced by FN (panels e-f), collagen type IV, the major basement membrane protein found at the epithelial-stromal interface (panel g), and Matrigel (panel h), which is a mixture of basement membrane proteins containing laminin, heparan sulfate proteoglycans, and entactin. As expected, adhesion to FN induced the γ -H2AX signals, whereas plating HCT116 cells on type-IV collagen or Matrigel did not significantly increase H2AX foci formation (panels g and h). Our data indicate that H2AX phosphorylation is not an obligatory consequence of cell-matrix interactions, but rather a specific response of cell adhesion to fibronectin. The intensity of γ -H2AX signals paralleled that of the FN concentrations ranging from 0.1 to 10 mg/ml (bottom line). Most cells presented high and medium γ -H2AX staining ($45 \pm 1.6\%$), which was maximal at the concentration of 10 $\mu\text{g/ml}$ FN (Fig. 1B).

FN promotes H2AX phosphorylation in a cell cycle and $\alpha 5$ integrin-dependent manner

Next, we examined the impact of FN on the cell division cycle in relation to γ -H2AX formation. Biparametric analysis of cell cycle progression and γ -H2AX signals was therefore performed by flow cytometry in HCT116 human colon cancer cells cultured on BSA and FN. As shown in Fig. 2A, FACS analysis showed that cell adhesion to FN is associated with an increase in the percentage of HCT116 cells into the S and G2/M phases of the cell cycle. A concomitant reduction in the percentage of G0/G1 cells was observed. Consistently, our data reveal that HCT116 cells cultured on FN-coated plates grew faster than those cultured on BSA-coated plates, according to their respective doubling time of 16 and 32 h. Since FN initiates intracellular signals through the canonical FN receptors $\alpha 5\beta 1$ integrins, we tested whether these ECM receptors are modulated by cell adhesion to FN. As shown in Fig. 2B, fibronectin selectively up-regulated the expression of $\alpha 5$ integrins in HCT116 colon cancer cells. Most interestingly, restitution of $\alpha 5$ by ectopic expression in $\alpha 5$ -deficient HT-29 cells potentiated the FN-induced γ -H2AX signals (Fig. 2B).

Implication of fibronectin in the activation of the DNA damage response pathways

By FACS analysis, we did not observe any increase in HCT116 cells at the sub-G1 fraction after plating cells on FN (data not shown). Thus, FN-induced H2AX phosphorylation was not linked to apoptotic DNA fragmentation, but more likely associated with early signaling events involved in DNA damage response. These findings point to a role of FN in the initiation of the DNA damage and repair signaling pathways. To test whether FN activates the PIKK-family members, we performed HCT116 immunostaining with antibodies against

phosphorylated forms of ATM Ser-1981 and p53 at Ser-15, a phosphorylation step involved in stabilization of p53 (Fig. 3A). Plating on FN led to positive staining of phospho-ATM and phospho-p53 in ~10% of the HCT116 cell population, consistent with PIKK activation. To further demonstrate the role of PIKK in FN-induced H2AX phosphorylation, we treated the cells with kinase inhibitors targeting PIKK family members, including the general PIKK inhibitor wortmannin, as well as selective inhibitors of DNA-PK (NU7026) and ATM (KU55933). As shown in Fig. 3B, wortmannin (20 μM) reduced by 80% the intensity of the γ -H2AX signals in cells plated on either BSA or FN, as quantified by digital imaging of pixel values within the nuclear volume. Treatment with KU55933/ATM and NU7026/DNA-PK inhibitors (each at 10 μM) also reduced the intensity of the FN-induced γ -H2AX nuclear foci by 40%, while the simultaneous addition of both inhibitors alleviated most of γ -H2AX signals. These data indicate that both ATM and DNA-PK are required for FN-induced H2AX phosphorylation. In addition, we observed that the protein kinase inhibitor SP600125 targeting JNK2 (10 μM) reduced H2AX phosphorylation in response to FN in HCT116 cells (not shown). Recent results indicate that absence of the stress activated protein kinase/c-Jun N-terminal kinase 2 (JNK2) is associated with reduced DDR (25).

Fibronectin and cisplatin mediate CHK2 and H2AX phosphorylation

We have shown that both ATM and DNA-PK are required in FN-induced H2AX phosphorylation. Therefore, we examined the impact of FN and cisplatin on phosphorylation levels of CHK2 at Thr⁶⁸ as this phosphorylation site is regulated by PIKK and is induced by genotoxins (26,27). In addition, we explored the correlation between CHK2-Thr⁶⁸ and H2AX phosphorylation in HCT116 colon cancer cells treated by FN and the genotoxin cisplatin (Fig. 4). By Western blotting and densitometric analysis, we detected a modest but significant elevation in the overall levels of γ -H2AX (15 kDa) after plating the cells on FN versus BSA (25% increase, $p < 0.04$), while the 25 kDa band detected in the presence of cisplatin is a polyubiquitinated form of the γ -H2AX protein induced by the recently identified E3 ubiquitin ligase (Ubl) RNF168 (28). Ubiquitination of histone H2AX is crucial to recruit important DDR mediators like the p53-binding protein 1 (53BP1) and BRCA1 to promote signaling responses elicited by DNA lesions. Upon DNA lesions, the chromatin modifier RNF168 Ubl is recruited and colocalized with γ -H2AX to DNA damage response foci where it contributes to increased ubiquitination of chromatin-associated proteins at DNA lesions, thereby facilitating downstream DDR signaling cascades (28-30). In comparison, BSA-treated HCT116 cells exposed to the genotoxin cisplatin showed a robust 2.3-fold increase in γ -H2AX levels at the 15 kDa band, together with induction of the 25 kDa phosphorylated band. Most interestingly, plating on FN potentiated 1.7 and 5-fold cisplatin-induced H2AX phosphorylation at the 15 and 25 kDa immunoreactive bands ($p < 0.03$ and $p < 0.01$, respectively). Consistent with the ability of FN to activate PIKKs, FN increased 9 and 5.6-fold the CHK2- Thr⁶⁸ phosphorylation levels (62 and 67 kDa bands) measured in control and cisplatin-treated HCT116 cells ($p < 0.001$ and $p < 0.008$), respectively.

Adhesion of human colon tumor stromal myofibroblasts to fibronectin leads to enhanced proliferation and potentiation of cisplatin-induced γ -H2AX formation

As shown in Fig. 5, cell proliferation was significantly increased 1.7-fold by FN in colon tumor stromal myofibroblasts in primary culture ($p < 0.001$). Interestingly, γ -H2AX

phosphorylation was not further increased by adhesion of myofibroblasts to FN, whereas cisplatin enhanced 4-fold control levels of γ -H2AX phosphorylation detected in BSA-treated myofibroblasts ($p < 0.002$). In addition, we observed that FN potentiated 2.6-fold the γ -H2AX phosphorylation levels induced by cisplatin in this model ($p < 0.001$).

Discussion

Activation of the DNA damage and repair response pathways can promote either cell survival or death depending on the extent of damage caused by oxidative stress, mutagens and chemoradiotherapy (31). Dysregulation of these mechanisms contributes to genomic instability, DNA damage tolerance, cancer initiation and progression.

In this report, we present evidence that adhesion to FN is sufficient to promote and to intensify cisplatin-induced DNA damage response (DDR) pathways that converge to cell cycle arrest, DNA repair and cell death. The formation of γ -H2AX foci has been detected in pre-cancerous lesions in epithelial tissues such as the bladder and colon but not in cancerous tissues upon progression of the disease (32). This observation suggests that the constitutive DDR pathways are already functional at early stages of carcinogenesis but are compromised upon tumor progression. Therefore, FN might contribute to the fine-tuning of the DDR pathways to promote DNA repair or apoptosis during the development, to compensate genomic instability during cancer progression, and to potentiate DNA and cellular damage in response to stress situations and exposure to genotoxic anticancer drugs. Here, we explored the early phases of the DDR pathways targeting the DNA damage sensor and molecular adaptor γ -H2AX in response to adhesion to FN in human colon cancer cells and tumor-derived myofibroblasts in primary culture. Phosphorylation of histone H2AX at Serine 139 (γ -H2AX) is one of the earliest signatures of the DDR and is required for the subsequent recruitment and retention of DNA repair factors. We showed that adhesion of human colon cancer cells to FN promoted phosphorylation of the DDR kinase CHK2 and p53. These two tumor suppressors orchestrate DNA repair and G2 arrest in response to cisplatin (9). Our data are consistent with the notion that plating cells on FN stimulates the PIKK family that includes ATM and DNA-PK. These kinases are involved in establishing and maintaining the G2 arrest induced by DNA damage response (33). In addition, H2AX may contribute to the fidelity of the mitotic process, even in the absence of DNA damage (34). The proliferation response induced by FN in human colon tumor stroma myofibroblasts is not related to increased γ -H2AX signals since intrinsic DNA damage is likely to be relatively limited in this model. Low proliferation rates are observed in control myofibroblasts (doubling time 40 h) as compared to HCT116 colon cancer cells (21 h). Therefore, the cytotoxicity of cisplatin in myofibroblasts appears also very limited in BSA-coated dishes according to the corresponding modest but significant inhibition of cell proliferation (20%, $p < 0.04$) although cisplatin treatment resulted in higher intensity of the γ -H2AX signals. Remarkably, adhesion to FN potentiated γ -H2AX expression in myofibroblasts treated by cisplatin, leading to 50% inhibition of cell proliferation by the genotoxin. Our data support the conclusion that the cisplatin-induced γ -H2AX signals correlate with the cytotoxicity of the DNA damaging agent in actively growing myofibroblasts plated on FN versus BSA. Thus, FN potentiates the genotoxicity of cisplatin through the DDR pathway sensor γ -H2AX. Coherently, we demonstrated that γ -H2AX

signals are also correlated with colon cancer cell proliferation following adhesion to FN. Alternatively; our data indicate that the canonical DDR pathways can be primed and re-activated in cancer cells by exposing them to the ECM component FN.

Down-regulation and loss of the integrin $\alpha 5\beta 1$ subunits are reported during human colon cancer progression (35-38) and in $\alpha 5$ integrin-deficient Caco-2 and HT29 cells (21). Consistently, we have shown that $\alpha 5$ integrins are up-regulated by adhesion to FN in HCT116 colon cancer cells, suggesting the implication of a self-activation loop between this ECM component and its canonical $\alpha 5/\beta 1$ receptors. In agreement, human cancer epithelial cells MCF-7 undergoing EMT and FN induction are associated with overexpression of $\alpha 5$ integrin transcripts (39,40). Furthermore, we have shown that expression of ectopic $\alpha 5$ is required for the induction of persistent and robust γ -H2AX signals by FN in $\alpha 5$ -deficient HT-29 colon cancer cells. This self-activation loop using the FN- $\alpha 5$ integrin connection is further supported by the progressive and dose-dependent induction of the γ -H2AX signals by FN in HCT116 colon cancer cells. In contrast, FN induced H2AX activation independently of the p53 status in human colon cancer cells HCT116 (p53-proficient) and HT29 (p53-deficient). However, little is known about the mechanisms regulating the expression, stability and degradation of FN (41). FN and its canonical $\alpha 5\beta 1$ integrin receptors activate transcriptional responses linked to the AP-1 complex (42). Interestingly, the AP-1 response element was also identified in the $\alpha 5$ promoter (43). Recent studies indicate that the PI3K/Akt and mTOR pathways control the expression of FN (44).

The relative contribution of FN and integrins to cancer cell survival, tumor growth, differentiation, and chemotherapy is still a matter of controversy. Both tumor suppression and progression responses have been described. Integrin engagement by ECM components mediates signaling cascades and crosstalk that are shared with certain elements of the canonical DDR, including PI3-kinase family, AKT, mTOR, the mitogen-activated and stress-activated kinases ERK, JNK2 and p38. These pathways are also connected with cancer cell proliferation, survival and metastatic responses (25,45-47). Consistently, FN and integrin $\alpha 5\beta 1$ are suspected to determine malignant phenotypes in human colon cancer and other epithelial tumors in breast and lung. However, opposite signals and behavior including suppression of anchorage-independent growth may originate from the main FN receptor $\alpha 5\beta 1$, FN variants and expression of other types of integrins, such as αv subfamily, $\alpha 8\beta 1$, $\alpha 4\beta 1$ and $\alpha 9\beta 1$. Genetic and hormonal factors inherent to the diversity of the genetic and molecular mechanisms driving colon cancer progression and the cellular context are also involved (35,48-50). For example, FN and $\alpha 5\beta 1$ integrins were shown to mediate proliferative signals via EGFR activation but tumor suppressor activity through ERBB2 inhibition (35,36,50). Thus, additional studies on integrated systems should redirect our attention in order to reconsider the complex interactions between cancer cells, cell adhesion receptors, and adjacent stromal cells that are representative of the tumor microenvironment during chemotherapy. It is well established that tumor stromal myofibroblasts play critical roles during cancer progression and modulate the therapeutic activity of anticancer drugs (22,51,52). Other components of the tumor stroma, including hypoxic endothelial and immune cells during tumor angiogenesis and inflammatory situations associated with colon cancer progression are also concerned (17,25,50).

In conclusion, our data establish for the first time a functional link between the cell adhesion molecule FN and the DNA damage response pathways. Our findings provide evidence that both colon cancer cells and tumor stroma myofibroblasts are ultimately targeted by the FN-dependent signaling cascades and γ -H2AX signals in response to the DNA damaging agent cisplatin. One can predict that high levels of FN and/or γ -H2AX foci in primary and metastatic tumors may indicate a more favorable response to genotoxic drugs, as recently shown for the ECM component SPARC in head and neck cancer patients (52,53). Thus, targeting FN and γ -H2AX-positive tumor cells appears as a promising strategy to improve the therapeutic activity of genotoxins involved in the initiation of the DDR pathways and cell death through the γ -H2AX adaptor and executor. Since the aging process is a critical factor to establish predisposition to the malignant transformation, additional understanding of the mechanisms underlying the connections between FN and the DDR pathways during aging and tumor evolution will shed light on the application of FN and FN-regulatory agents in DNA repair, cancer prevention and therapy.

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Abbreviations

ATM	ataxia telangiectasia mutated
CHK2	checkpoint kinase 2
DNA-PK	DNA-dependent protein kinase
ECM	extracellular matrix
FACS	fluorescence-activated cell sorter
FAK	focal adhesion kinase
γ-H2AX	Ser139 phosphorylated histone H2AX
JNK2	c-Jun-NH2-terminal kinase-2
PIKK	phosphatidylinositol-3-kinase-related protein kinases

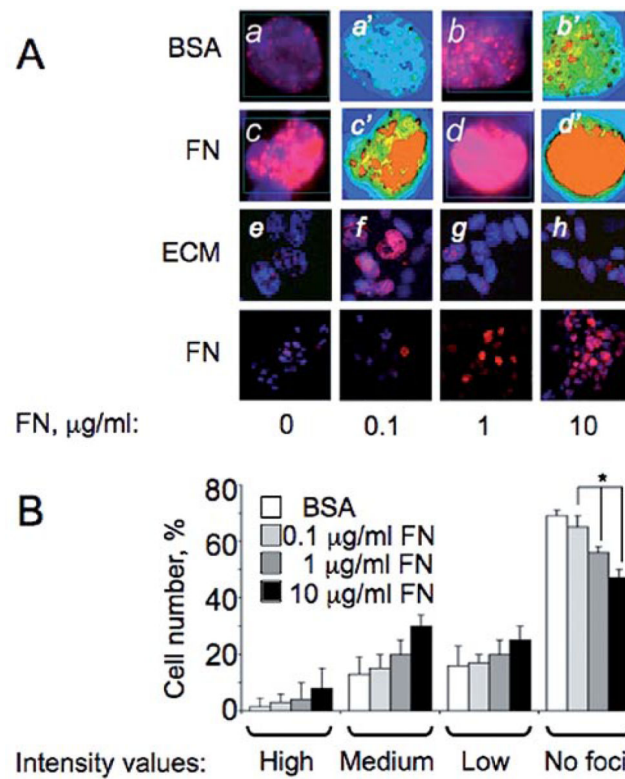
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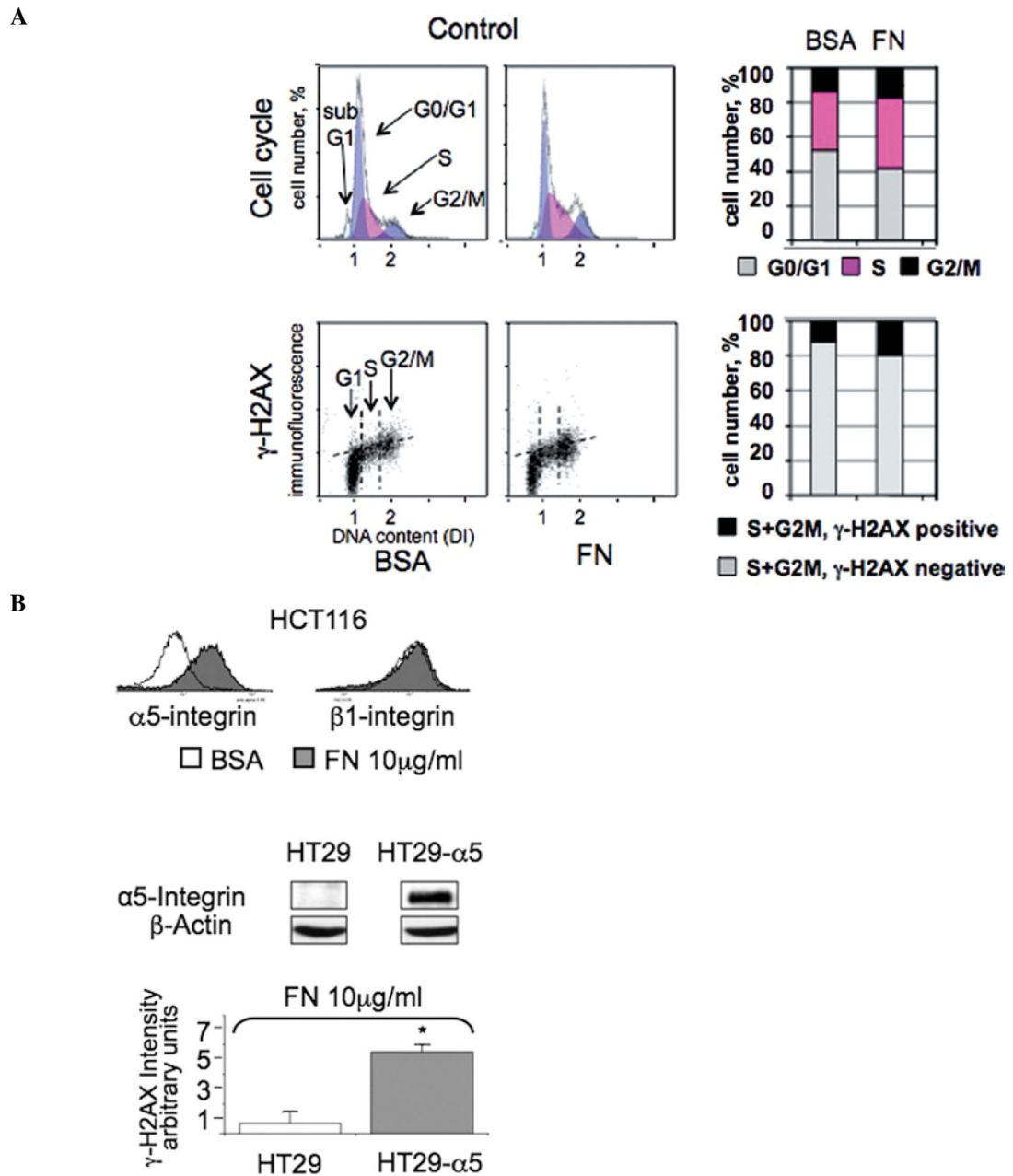
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**Figure 1.**

Staining and intensity maps of fibronectin-induced Ser139 H2AX phosphorylation signals as nuclear γ -H2AX foci in HCT116 colon cancer cells. (A) Fluorescence micrographs of Ser139 H2AX (γ -H2AX) nuclear foci in HCT116 cells following 24 h adhesion to BSA (panels, a-b) and fibronectin (panels c-d, 10 μ g/ml FN). γ -H2AX expressing cells were detected and stained with the γ -H2AX Texas red-tagged antibody 594 (red fluorescence), combined with DAPI at 617 nm for nuclei staining in blue. For immunocytochemistry, HCT116 cells were fixed with 4% formaldehyde in PBS. Original magnification, x200. Intensity of the γ -H2AX signals was quantified by the corresponding density maps (a'/b' and c'/d') using the automated deconvolution three-dimensional (3D)-digital image microscope system (Applied Precision, Issaquah, WA). Panels e-h: Impact of FN and extracellular matrix (ECM) components on γ -H2AX levels. Fluorescence micrographs of γ -H2AX levels in HCT116 colon carcinoma cells cultured for 24 h on 1% BSA (control, panel e), fibronectin (FN, panel f), Matrigel (g), and collagen type IV (h), each at 10 μ g/ml. Original magnification, x100. Data are representative of 4 independent experiments. Bottom: Effects of 1% BSA (control) and FN at concentrations ranging from 0.1 to 10 μ g/ml on the γ -H2AX signals. (B) Data were quantified as γ -H2AX signals (high, medium to low intensity levels, and no signal recorded: no foci). A series of optical sections along the z-axis was acquired with increments of 0.2 μ m using oil immersion objectives. The data set were deconvolved and analyzed to generate three dimensional/volume or section views. Data are representative of 10 independent experiments.

**Figure 2.**

Implication of the cell proliferation cycle and $\alpha 5$ integrins in fibronectin-induced γ -H2AX signals in HCT116 and HT29 human colon cancer cells. (A) Upper panel: cells cultured for 48 h on BSA or fibronectin (FN) were in G1, S, and G2/M phases of the cell cycle as indicated, with a small percentage (less than 6%) in the sub-G1 fraction. Histograms show the distribution of cells in the different phases of the cell cycle based on DNA fluorescence intensity (DNA content): G0/G1 (grey), S (pink) and G2/M (black). Lower panel: scatter plots show the bivariate analysis of γ -H2AX immunofluorescence and DNA content. The dashed lines represent the threshold of γ -H2AX positivity and the range of cells in G1, S and G2/M as indicated. Of note, only γ -H2AX-positive cells with high and medium γ -

H2AX signal intensity were detected and mainly observed at the S and G2/M phases by FACS analysis, as compared to the immunofluorescence study shown in Fig. 1. Data are representative of 3 experiments. (B) Upper panel: plasma membrane targeting of $\alpha 5$ integrins by FN in human colon cancer HCT116 cells. Flow cytometric analysis of $\alpha 5$ and $\beta 1$ integrin subunit expression. Non-specific IgG was used as negative control. Lower panel: Western blot analysis of forced $\alpha 5$ integrin expression in HT29 colon cancer cells compared to $\alpha 5$ subunit-deficient HT29-pcDNA cells. Both control HT29 and HT29- $\alpha 5$ cells were plated on FN at 10 $\mu\text{g}/\text{ml}$ for 24 h. Ectopic expression of $\alpha 5$ integrins promotes γ -H2AX phosphorylation in HT29 cells plated on FN. Immunoblots of β -actin was used as control. Data are representative of three experiments.

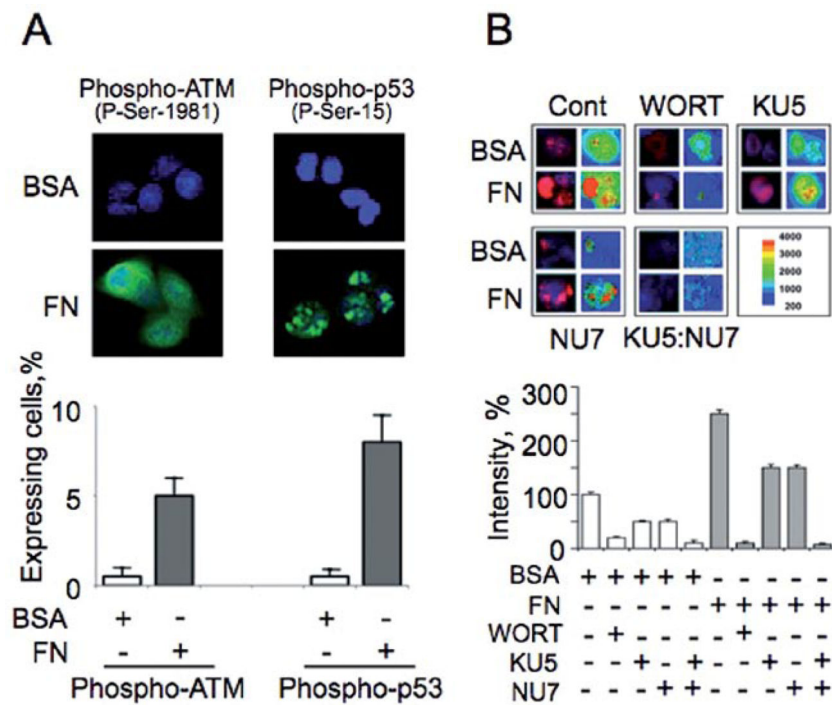
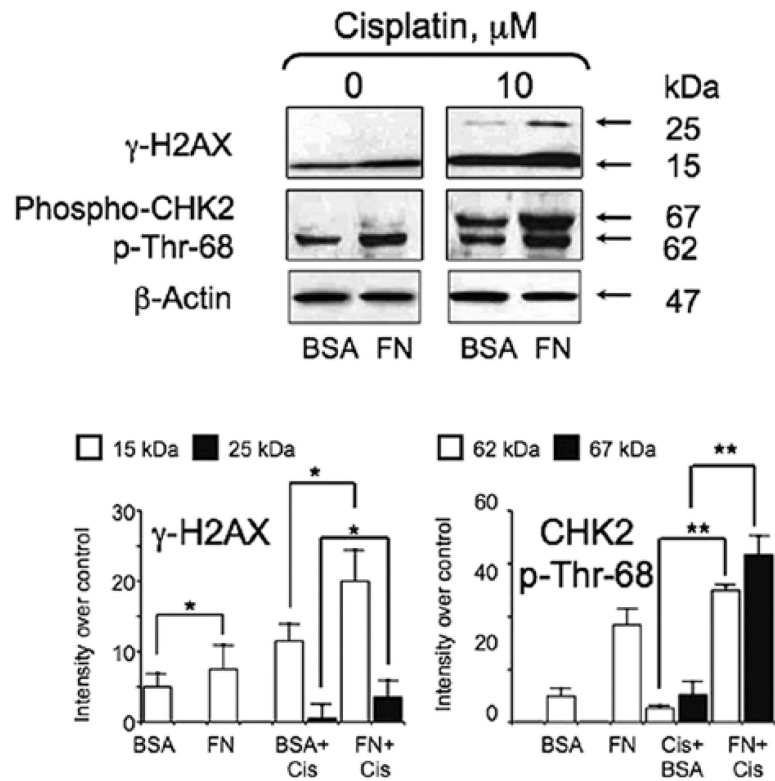


Figure 3.

Effect of kinase inhibitors targeting the DNA damage response pathways on fibronectin-induced H2AX phosphorylation. (A) Activation of the checkpoint-dependent signals ATM and p53 in HCT116 human colon cancer cells plated on FN. Upper panel: cells were plated onto coverslips pretreated overnight with 1% BSA (control) or 10 $\mu\text{g}/\text{ml}$ FN and processed for immunocytochemistry using the mAbs against the phosphorylated forms of ATM at Ser-1981 and p53 at Ser-15. DNA staining with DAPI is shown in blue. Lower panel: representative histograms of phospho-ATM and -p53 positive HCT116 cells plated for 24 h on FN. Original magnification, x63. Data are representative of 3 separate experiments. (B) Control and FN-induced H2AX phosphorylation require ATM and DNA-PK. Upper panel: HCT116 cells were cultured for 24 h onto 1% BSA or 10 $\mu\text{g}/\text{ml}$ fibronectin-pretreated glass coverslips in the presence and absence of pharmacological inhibitors targeting PIKK (wortmannin: WORT, 20 μM), ATM (KU55933: KU5, 10 μM), and DNA-PK (NU7026: NU7, 10 μM), either alone or combined, as indicated. Cells were stained with the anti-human γ -H2AX antibody, followed by Texas red 594 secondary mAb. Lower panel: quantitative analysis of γ -H2AX immunofluorescence intensity in HCT116 cells plated on BSA or FN. Original magnification, x100. Data are representative of 5-10 separate experiments.

**Figure 4.**

Convergence between fibronectin and the DNA damaging agent cisplatin at $\gamma\text{-H2AX}$ foci signaling in human colon cancer cells HCT116. (A) Immunoblots and quantification of $\gamma\text{-H2AX}$ phosphorylation (25 and 15 kDa bands), phospho-CHK2-Thr⁶⁸ (67 and 62 kDa bands), and $\beta\text{-actin}$ as a loading control. (B) Quantification of the relative intensity of the corresponding immunoreactive bands representing phosphorylated forms of $\gamma\text{-H2AX}$ and CHK2. Data are representative of three experiments; statistically significant at * $p < 0.04\text{-}0.01$ and ** $p < 0.008\text{-}0.001$ from the respective controls, as indicated.

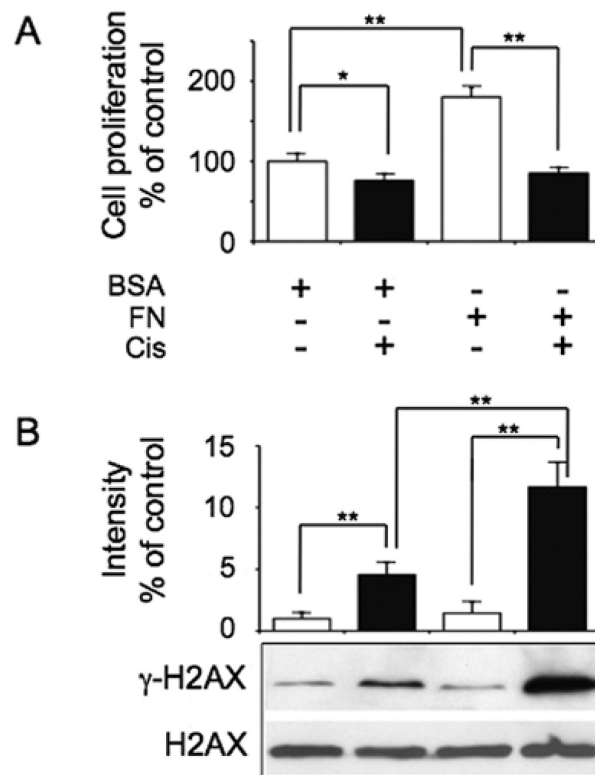


Figure 5.

Impact of fibronectin on the cytotoxicity of cisplatin and activation of H2AX in human colon myofibroblasts isolated from clinical tumor specimen. Human colon-derived primary myofibroblasts were isolated and cultured from tumor tissue, as described (22). Cells were allowed to adhere to BSA and FN for 72 h. (A) Cell proliferation was determined by the MTT test under control conditions (BSA, 100%) and after treatment with cisplatin (Cis, 10 μ M) for 48 h, as indicated. (B) Immunoblot analysis of the γ -H2AX signals recorded in the corresponding control and treated myofibroblasts. Results are the means \pm SE from 3 experiments. Significantly different at * $p < 0.04$ -0.02 and ** $p < 0.002$ -0.001 from the respective controls.