NF-κB Regulates Radioresistance Mediated By β1-Integrin in Three-Dimensional Culture of Breast Cancer Cells

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Abstract

β1-integrin induction enhances breast cancer cell survival after exposure to ionizing radiation (IR), but the mechanisms of this effect remain unclear. Although NF-κB initiates prosurvival signaling pathways post-IR, the molecular function of NF-κB with other key elements in radioresistance, particularly with respect to extracellular matrix-induced signaling, is not known. We discovered a typical NF-κB-binding site in the β1-integrin promoter region, indicating a possible regulatory role for NF-κB. Using three-dimensional laminin-rich extracellular matrix (3D lrECM) culture, we show that NF-κB is required for β1-integrin transactivation in T4-2 breast cancer cells post-IR. Inhibition of NF-κB reduced clonogenic survival and induced apoptosis and cytostasis in formed tumor colonies. In addition, T4-2 tumors with inhibition of NF-κB activity exhibited decreased growth in athymic mice, which was further reduced by IR with downregulated β1-integrin expression. Direct interactions between β1-integrin and NF-κB p65 were induced in nonmalignant breast epithelial cells, but not in malignant cells, indicating context-specific regulation. As β1-integrin also activates NF-κB, our findings reveal a novel forward feedback pathway that could be targeted to enhance therapy.

Introduction

Aside from well-known genetic and epigenetic alterations, increasing evidence suggests that microenvironmental factors substantially contribute to acquired or developed cancer therapy resistance (1, 2). Integrins, a family of transmembrane cell surface receptors, are composed of α and β subunits, that critically mediate cell–extracellular matrix (ECM) interactions (3). β1-integrins are aberrantly expressed in human breast carcinomas and play multifaceted roles in determining cell fate, effecting cell survival, proliferation, apoptosis, invasion, metastasis, and tissue organization (4, 5). Similar to other integrin receptors, β1-integrins are overexpressed in various cancers, including breast cancers, and have been shown to mediate tumor cell resistance to chemotherapy (6, 7) and radiation therapy (8, 9). Therefore, understanding β1-integrin-mediated signaling is crucial for the optimization and development of innovative chemo- and radiotherapeutic approaches.

Several experimental models have shown the efficacy of β1-integrin inhibitors to inhibit metastasis in colon and breast models, refractory tumors, and advanced metastatic disease (10–12). Targeting of β1-integrin has also shown strong potential to sensitize cancer cells to conventional radiotherapies and chemotherapies (13–15). In addition to preclinical studies, clinical trials evaluating β1-integrin antagonists are still ongoing. To date, 3 β1-integrin inhibitors have been or are being evaluated in clinical trials: ATN-161, volociximab (M200), and JSM6427. In contrast to these anti-integrin monotherapies that target specific α/β integrin heterodimers and have only been tested as monotherapy so far and with limited efficacy, A1IB2 (β1-integrin function–blocking antibody), used in this study, may be more effective as it targets multiple integrins simultaneously. However, the underlying molecular mechanisms of β1-integrin-mediated resistance to radiotherapy remain largely unclear.

NF-κB, a stress-sensitive heterodimeric transcription factor in the regulation of the stress-responsive genes, is activated upon phosphorylation and proteolysis of 1κB or by an 1κB-independent pathway (16). Tumor cells express high levels of constitutive NF-κB activity (17), leading to increased cell survival via antagonism of apoptotic pathways (18). NF-κB has been directly implicated in the cellular resistance to radiation and chemotherapy (19). IR-induced NF-κB activity is associated with enhanced survival in human leukemic K562 cells (20) and papillomavirus-transformed human keratinocytes (21). In addition, the link between constitutive NF-κB activity, basal apoptosis, and radiosensitivity has been reported in breast carcinoma cell lines (22, 23). Thus, the elevated NF-κB activity identified in human tumors may lead not only to apoptosis suppression but also to radiotherapy.
resistance. However, the cooperative function of NF-kB with other key stress elements in radiosensitivity remains to be elucidated.

Recently, we discovered that a typical binding site for NF-kB was located in human β1-integrin promoter. In our previous studies, we have shown that downmodulation of β1-integrin, via inhibitory monoclonal antibody AIIB2, effectively synergizes with IR to modify Akt-mediated radiosensitivity in breast cancer cell lines in three-dimensional laminin-rich extracellular matrix (3D IrECM) cell culture model (8, 24). In addition, we showed that AIIB2 dramatically enhanced radiotherapy efficacy in human breast cancer xenografts (13). Thus, we hypothesized that β1-integrin pathways in radiation-induced cell death in malignant breast cells in an in vitro 3D culture and tumor growth in vivo. Here, we show that β1-integrin inhibition, radiosensitization is regulated by NF-kB via increased transcriptional activity, and a loop-like β1-integrin–NF-kB–β1-integrin pathway is activated post-IR. Our results suggest a promising approach to radiosensitize malignant breast cancers by targeting NF-kB/β1-integrin pathways.

Materials and Methods

Cell culture

The isogenic cell lines, nonmalignant S1 and malignant T4-2 cells, from the HMT3522 human breast cancer progression series were maintained as described previously (25). The cell series was established in an attempt to recapitulate the stochastic and prolonged nature of breast cancer progression by continuously culturing S1 cells, derived from reduction mammoplasty, in the absence of serum followed by EGF removal and injection into mice, to give rise to T4-2 cells (26, 27). The S1 cells were propagated as monolayers on plastic in the presence of 10 mg/mL EGF (BD Biosciences); the T4-2 cells were grown as monolayers on dishes coated with collagen type I (Vitrogen 100, Celtrix Laboratories) in the absence of EGF. Three dimensional (3D) cultures were prepared by growing S1 and T4-2 cells to confluence as monolayers, followed by trypsinization and seeded as single cells (8.5 × 10^5 cells/mL) at the density of 2.5 × 10^4 cells/mL and 1.8 × 10^4 cells/mL, respectively, into EHS matrices (Trevigen). Human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection, and maintained in Dulbecco’s Modified Eagle Medium (DMEM/F12; UCSF Cell Culture Facility, San Francisco, CA) with 5% FBS and penicillin/streptomycin.

Apoptosis and proliferation assays

Apoptosis and proliferation were detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and indirect immunofluorescence of Ki-67 nuclear antigen, respectively, in samples taken from 3D IrECM at day 6 (MCF-7) and 7 (T4-2 and MDA-MB-231; Figs. 2A and 4A) as described previously (28).

Confocal microscopy

Confocal images were acquired by using a Zeiss LSM 710 inverted laser scanning confocal microscope equipped with an external argon laser. Using a Zeiss Fluo 40×W (1.3 numerical aperture) objective, images were captured at the colony midsection. Relative immunofluorescence intensity of images was standardized by comparing only cultures that were processed identically and stained in the same experiment.

Other materials and methods

Radiosensitivity assay, Western blotting, immunofluorescence, NF-kB DNA-binding assays, real-time PCR analysis, immunoprecipitation, in vivo caspase-3/7 activity assay, tumor inhibition assay in nude mice, immunohistochemistry stainings, and statistics are described in the Supplementary Information section.

Results

NF-kB inhibition sensitizes human malignant breast cancer T4-2 cells to ionizing radiation

We have previously shown that malignant T4-2 colonies are significantly more resistant to radiation-induced death compared with nonmalignant counterpart S1 acinar structures in 3D IrECM (13). We used clonogenic survival assays to verify that malignant T4-2 cells indeed had increased reproductive capacity compared with S1 cells post-IR (Fig. 1A). In addition, β1-integrin inhibition by AIIB2 monoclonal antibody increased radiosensitivity in T4-2 cells (Fig. 1B). It is well documented that NF-kB is activated by IR and plays a central role in radiation resistance (23, 29). The activity of NF-kB is largely regulated by its subcellular localization. Using immunofluorescence for p65 (a major subunit of NF-kB), we measured the effect of IR on the activation of NF-kB in T4-2 and S1 cells in 2-dimensional (2D) monolayer culture. In S1 cells, 4-Gy IR rarely induced nuclear translocation of p65. In contrast, nuclear p65 was observed in 8.6% of Sham-irradiated T4-2 cells and 59% of T4-2 cells exposed to 4-Gy at 4 hours (Figs. 1C and D). Using a microwell colorimetric assay, we also found that NF-kB DNA-binding activity was significantly increased in a time-dependent manner in T4-2, but not S1, cells after exposure to 4-Gy X-ray. To determine the binding specificity, oligos with intact binding site for NF-kB (wild-type) or the mutated site (mutated) as a competitor for p65 binding to the NF-kB consensus sequence were used. The addition of a 10-fold molar excess of the wild-type oligo significantly competed for binding of p65 to the NF-kB consensus sequence; however, no competition was observed with a 10-fold molar excess of the mutated oligo (Fig. 1E). In addition, using TFBIND software, we discovered that an NF-kB–binding site (ggagagcccg-96 to -87) was located in the human β1-integrin promoter region (Fig. 1F). These results indicated that β1-integrin–mediated radiosensitivity of T4-2 cells is regulated by NF-kB. If NF-kB positively regulates β1-integrin, inhibition of NF-kB activity should increase radiosensitivity. To test this, NF-kB activity was inhibited by 4-Methyl-N-(3-phenylpropyl)benzene-1,2-diamine (JSH-23) and then colony formation assay was conducted. As shown in Fig. 1B, JSH-23 resulted in a significant decrease in surviving clones. Overall, these findings show that β1-integrin is a novel target of
NF-κB in radiosensitivity of breast cancer cells, but not non-malignant human mammary epithelial cells.

**NF-κB activation is required for radiation-induced β1-integrin overexpression**

In a 3D laminin-rich ECM (lr-ECM)–based cell culture model (Fig. 2A; experimental schema), which better mimics physiologic growth conditions than 2D cultures (8, 30–32), malignant breast cancer T4-2 cells showed higher levels of total and phospho (T788/789) β1-integrins compared with its counterpart nonmalignant S1 cells (Fig. 2B). These protein levels were further induced in T4-2 cells post-IR (4-Gy X-ray; Fig. 2C). Interestingly, treatment of T4-2 colonies, formed at day 4, with NF-κB inhibitors JSH-23 or IMD-0354, resulted in a decrease in phospho and total β1-integrins, which was further reduced by IR (Fig. 2C and Supplementary Fig. S1A). At 30 μmol/L concentration of JSH-23, which selectively blocks nuclear translocation of NF-κB p65 and its transcription activity, both phospho and total β1-integrins were significantly reduced independently of radiation exposure. (Fig. 2C). IMD-0354, an IKK-β inhibitor that blocks IκB-α phosphorylation and NF-κB availability, also significantly reduced phospho and total β1-integrins by 5 μmol/L and 10–20 μmol/L, respectively, with or without IR (Supplementary Fig. S1A). Similarly, IR-induced increases in β1-integrin mRNA expression were significantly reduced by JSH-23 in a dose-dependent manner (Fig. 2D).
confirm inhibition of β1-integrin activity, phosphorylation of FAK397, a critical indicator of downstream β1-integrin signaling, was measured. As shown in Supplementary Fig. S1B, inhibition of β1-integrin by NF-κB inhibitor JSH-23 significantly reduced the levels of total and phosphorylated FAK. These results clearly show that IR-induced β1-integrin expression and its downstream signaling are mediated by NF-κB. Similar to β1-integrin, protein levels of p65/p50, the major inducible NF-κB dimer, were also higher in T4-2 than S1 cells (Fig. 2B). We prepared whole-cell lysates for immunoblotting of p65 and p50 to determine whether NF-κB transactivation following IR was associated with an increase in p65/p50 levels. Compared with Sham (0 Gy) IR control cells, a clear increase in p65/p50 was observed post-IR; in addition, these levels were decreased by JSH-23 in a dose-dependent manner (Fig. 2E). Together with Fig. 2C, these results indicate that NF-κB and β1-integrin are components of a coordinated response to radiation.
Next, we asked whether transcriptional activity directed through NF-κB was involved in the upregulation of β1-integrin post-IR. To determine this, we conducted an ELISA-based DNA binding assay in 3D lrECM to quantify the binding of NF-κB to its binding site identified in the β1-integrin promoter (Fig. 1F). A 50 bp oligo containing the β1 binding site on the β1-integrin promoter region was synthesized and then used as a DNA probe to assess binding of IR-activated NF-κB in T4-2 and S1 cells. As shown in Fig. 2F, NF-κB DNA-binding activity was much higher (~4-fold) in T4-2 compared with S1 cells and 4-Gy X-ray significantly increased binding activity. However, the binding activities were not induced with or without radiation when a 50 bp DNA probe containing a mutation in the β1 site was used. To test the generalizability of these results, the induction of DNA binding activity of NF-κB observed in T4-2 cells post-4-Gy X-ray was confirmed in another cell line (Supplementary Fig. S1B). Similar to T4-2 cells, binding activities were significantly increased (~3.8-fold) post-IR in human malignant breast cancer MDA-MB-231 cells. As expected, this induction of DNA binding of NF-κB was abolished when the mutated probe was used. Together, these data identified β1-integrin as a downstream transcriptional target of NF-κB in 3D lrECM T4-2 cell cultures.

Inhibition of NF-κB activity reduces colony size via increasing apoptosis and decreasing proliferation in malignant breast cancer colonies in 3D lrECM

We showed previously that inhibition of β1-integrin by monoclonal antibody AIIB2 significantly increased TUNEL-positive nuclei and decreased Ki-67-positive nuclei in a panel of malignant breast cancer colonies (28). Figure 2 shows that NF-κB positively regulates β1-integrin, which suggests that NF-κB inhibition may reduce the colony size via increasing apoptosis and decreasing proliferation in malignant breast cells. To test this hypothesis, malignant T4-2 colonies and nonmalignant S1 acini on top of 3D lrECM gels with 5% Matrigel formed at day 4 and 6, respectively, were treated with NF-κB inhibitor JSH-23 and then exposed to sham or a single 4-Gy X-ray 24 hours after treatment. Upon NF-κB inhibition, T4-2 colony size was significantly reduced at 48 hours. IR further reduced the size in a dose-dependent manner (data for 20 and 30 μmol/L were not shown; Figs. 3A and B). The maximum size difference was reached in colonies treated with 40 μmol/L JSH-23 and exposed to IR, associated with a substantial approximately 40% decrease in the average colony diameter. In contrast, the average diameter of S1 acini was only marginally reduced by 20–40 μmol/L JSH alone, and no further reduction was observed by the addition of IR (data for 10 and 30 μmol/L were not shown; Supplementary Figs. S3A and S3B).

Next, to correlate the decrease in colony size by JSH-23 with apoptosis and proliferation in malignant breast cells, immunofluorescence (IF) staining for TUNEL and caspase-3/7 activity assay, and IF staining for Ki-67 nuclear antigen, respectively, were conducted. Consistent with the decrease in size of T4-2 colonies by JSH-23 as described above, TUNEL-positive nuclei were significantly increased in sham-treated colonies and a further induction was observed after IR exposure (Figs. 3A and C). At the concentration of 40 μmol/L JSH, a 5.8 times increase in TUNEL-positive nuclei was observed compared with control cells post-IR (8% versus 49%; Fig. 3C). To verify IR- and JSH-23-induced apoptosis, we conducted a caspase-3/7 activity assay (Fig. 3D). A 9-fold induction of caspase activity was observed by JSH-23 (40 μmol/L) alone, which was further increased with IR. The combined treatment of JSH-23 or β1-integrin inhibitory AIIB2 monoclonal antibody with IR was associated with the highest caspase activity compared with either single agent used alone. In addition, we analyzed the effect of caspase inhibition by applying the pan-caspase inhibitor Z-VAD-FMK and found that Z-VAD-FMK reversed apoptosis mediated by NF-κB inhibition with or without IR, further supporting the finding that NF-κB inhibition induces apoptosis of T4-2 cells. In addition, the induction of apoptosis by JSH-23±IR was associated with a decrease in proliferation. A significant decrease in the percentage of Ki-67-positive cells with JSH-23 treatment was observed in sham IR cells, which was further reduced by IR in a clearly dose-dependent manner (data for 20 and 30 μmol/L were not shown; Figs. 3A and E). The maximum reduction of proliferation was observed in T4-2 colonies treated with 40 μmol/L JSH-23 compared with control cells post-IR (33% versus 11%; Fig. 3E). Together with Fig. 2, these results provide strong evidence that β1-integrin–induced resistance to IR is regulated by NF-κB.

To test whether the increase in radiosensitivity by NF-κB inhibition is a common mechanism for other malignant breast cancer cell lines, MDA-MB-231 and MCF-7 cells were seeded in 3D lrECM (experimental schema is shown in Fig. 4A) to examine the percentage of TUNEL- and Ki-67–positive cells with JSH-23 treatment with and without IR. As shown in Figs. 4B and D, the percentage of TUNEL–positive cells was significantly increased in both MDA-MB-231 and MCF-7 cells and further induced post-IR. We did find a differential sensitivity to JSH-23 among the cell lines. Notably, the level of apoptosis was lower in MCF-7 cells than T4-2 (34% versus 49%) and MDA-MB-231 (34% versus 44%) cells after treatment with 40 μmol/L JSH-23 post-IR, indicating less relative sensitivity in this cell line. To reach the percentage of apoptosis in MCF-7 cells that was similar to T4-2 or MDA-MB-231, a higher concentration of JSH-23 was required (Figs. 3C and 4B and D). Similar dose-dependent findings were reflected in the degree of cytostatic response, measured by the percentage of Ki-67–positive cells (Figs. 4C and E). Although we treated MCF-7 cells with higher concentrations of JSH-23 (60 and 80 μmol/L), the percentage of Ki-67–positive cells remained higher than T4-2 and MDA-MB-231 cells post-IR (Figs. 3E and 4C and E). These results indicate that β1-integrin may be differentially regulated by NF-κB among different malignant breast lines.

Interactions of β1-integrin with NF-κB p65 and α5-integrin are oppositely regulated in S1 and T4-2 cells post-IR

NF-κB physically and/or functionally interacts with many proteins involved in cell proliferation and survival (23). In light of our current results that IR-induced β1-integrin is regulated by NF-κB, we used immunoprecipitation to test the hypothesis that NF-κB physically interacts with β1 integrin. This showed that NF-κB p65 was able to interact
with β1-integrin in sham-irradiated S1 and T4-2 cells in 3D lrECM (Supplementary Figs. S4A and S4B). Because the protein levels of β1-integrin and p65 were high in T4-2 and further induced post-IR compared with S1 cells (Figs. 2B, C, and E), we expected that IR would increase β1-integrin/p65 interaction in T4-2 than S1 cells. However, we found that the interaction was strikingly absent post-IR in relatively resistant T4-2 cells and was enhanced in nontumorigenic S1 cells (Supplementary Figs. S4A and S4B; marked with a box). Together with Figs. 1A and B, these results indicate that NF-κB may inhibit β1-integrin function via physical interaction to increase radiosensitivity in nontransformed cells, but not in malignant cells. As we have shown previously (8), the interaction of β1-integrin with one of its heterodimeric partners α5-integrin was almost absent in S1, but very high in T4-2, cells without radiation. Although IR slightly induces α5β1-integrin complex in both S1 and T4-2 cells, the level of interaction was much higher in T4-2 than S1 cells post-IR.
Together with Supplementary Figs. S4A and S4B, these results suggest that the induction of physical interaction of \( \beta_1 \)-integrin with \( \alpha_5 \)-integrin, but not with p65, promotes radioresistance in malignant breast cancer cells in 3D IrECM. The exact mechanism underlying the interaction of \( \beta_1 \)-integrin with p65 and \( \alpha_5 \)-integrin in radiosensitivity is the subject of ongoing investigations.

**Inhibition of NF-κB activity results in tumor growth inhibition in vivo associated with downregulated \( \beta_1 \)-integrin expression**

To test whether the in vitro observations in 3D IrECM culture could be validated in vivo, we examined tumor growth from mice injected with vehicle or NF-κB–inhibited T4-2 cells exposed to sham or 4-Gy X-ray. As shown in the tumor growth curves (Figs. 5A and B), NF-κB inhibition by 20 μmol/L of JSH-23 in T4-2 cells (T4+JSH) significantly delayed the growth of tumors relative to control in athymic mice. We also found that tumors exposed to IR post-JSH-23 treatment (T4+JSH+IR) grew dramatically slower than control. The significant suppression of tumor formation was observed at day 13 after a subcutaneous injection of T4+JSH or T4+JSH+IR cells and continued till the day of sacrifice (day 28).

We next tested the expression levels of \( \beta_1 \)-integrin and NF-κB p65 by immunohistochemical staining (Fig. 5C). As seen in the representative tissue sections, \( \beta_1 \)-integrin was reduced in T4+JSH tumors and a severe reduction was observed in tumors formed by T4+JSH+IR. Similarly, total and nuclear NF-κB p65 were also reduced in T4+JSH and a further reduction was observed in T4+JSH+IR tumors. Then, Western blot analysis of tumor sections was conducted to further determine the expression levels of \( \beta_1 \)-integrin and NF-κB. The Western
data also revealed a decrease in phospho and total β1-integrins together with p65/p50 in NF-κB–inhibited T4-2 tumors, which was further reduced by IR (Fig. 5D). The results of these experiments show that NF-κB inhibition in T4-2 cells with or without IR decreases the tumor growth via inhibition of β1-integrin expression in athymic mice. Together with Figs. 2C–F, the data in Fig. 5 strongly suggest that β1-integrin expression is regulated by NF-κB.

Inhibition of β1-integrin reversed IR-induced NF-κB protein levels and DNA binding activity in 3D lrECM

The functional role of integrin-induced NF-κB in cell survival was first showed by Scatena M and colleagues in α5β3-integrin-mediated endothelial cells (33). To determine whether β1-integrin function blocking monoclonal antibody AIIB2 was able to inhibit IR-induced NF-κB p65/p50 expression and DNA-binding activity, immunoblotting for p65 and p50, and an ELISA-based DNA-binding assay were conducted in T4-2 cells in 3D lrECM. As shown in Fig. 6A, IR-induced p65/p50 protein levels were significantly reduced by 0.2 μg/μL AIIB2, suggesting that NF-κB is a downstream target of β1-integrin. Inhibition of β1-integrin also significantly reduced IR-induced NF-κB DNA-binding activity (Fig. 6B). Together with Figs. 1B and 2C and F, these results suggest a loop-like β1-integrin–NF-κB–β1-integrin pathway is activated post-IR to induce radioresistance in T4-2 cells in 3D lrECM (schematic presentation is shown in Fig. 6C).

Discussion

Cancer cells dynamically interact with their microenvironment to remodel the surrounding stroma and facilitate growth and invasion. Tumor ECM has been shown to increase resistance to cytotoxic cancer therapy including radiation (6, 7) and has also been associated with poor outcomes in subgroups of patients with breast cancer (34). However, the underlying basis for ECM-mediated resistance to therapy, particularly after radiation, has not been well studied. We have previously shown that targeting β1-integrin leads to selective apoptosis and cytostasis in breast cancer cells in vivo without toxicity. In addition, a number of
studies have shown that β1-integrins regulate radiation-induced prosurvival signaling, leading to increased survivability and reproductive capacity of human cancer cells exposed to ionizing radiation (8, 9, 13, 14, 28). In the present study, we wished to further dissect the possible molecular mechanisms associated with β1-integrin regulation of survival in irradiated cancer cells. We discovered that a typical NF-κB–binding site was located in the promoter region of the β1-integrin gene (Fig. 1F). Given the known role of NF-κB in mediating an acute phase stress response to IR, we sought to further investigate a potential novel relationship between ECM-directed signaling and β1-integrin-mediated survival. Here, we show that NF-κB directly modifies β1-integrin expression, in part through transcriptional activation (Figs. 2D and F). This is associated with increased survival post-IR, and sensitization of cells to IR-induced death upon inhibition of NF-κB (Figs. 1B and 3C and D). This pathway is functional in several breast cancer cell lines, and notably oppositely regulated in normal human mammary epithelial cells (Supplementary Fig. S3). Finally, inhibition of β1-integrin reverses NF-κB transcriptional activity (Fig. 6B), suggesting a loop-like β1-integrin–NF-κB–β1-integrin regulatory pathway in radioresistance of malignant breast cells in 3D ECM.

Figure 6. Inhibition of β1-integrin reversed IR-induced NF-κB expression and DNA-binding activity in 3D hECM. A, Western blot analyses on the expression of NF-κB p65/p50 and β1-integrin using whole-cell lysates prepared from malignant breast cancer T4-2 cells at day 7 in 3D hECM as shown in Fig. 2A. β-actin serves as an internal loading control. Right, relative expression levels of NF-κB p65/p50 normalized to the expression levels of β-actin. B, NF-κB DNA-binding assay using whole-cell lysates prepared from T4-2 cells as above. Data are mean ± SD of pooled results from 3 independent experiments (n = 3); **, P < 0.01. C, schematic representation of radiation-induced loop-like β1-integrin–NF-κB–β1-integrin pathway in radioresistance of malignant breast cells in 3D hECM.
in vivo, related to regulation of Akt signaling downstream of β1-integrin (8, 13). Cordes and colleagues showed a FAK/cortactin-dependent regulation directly linked of β1-integrin-mediated survival post-IR in squamous cell carcinoma of the head and neck (9). Thus, increasing evidence indicates that inhibiting β1-integrins enhance the therapeutic efficacy of radiotherapy through critical signaling pathways. However, modulation of gene expression is one of the most important events because it directly controls cellular adaptation to genotoxic conditions; yet little is known regarding the potential transcriptional mechanisms involved in the β1-integrin-dependent survival pathway of cancer cells after IR treatment. Here, we show that IR induces p65 translocation into the nucleus (Figs. 1C and D) and concomitantly increases NF-κB binding to the β1-integrin promoter region (86 bp upstream of transcription initiation site; Fig. 1E), suggesting that IR-induced NF-κB activity transcriptionally upregulates β1-integrin.

To elucidate the functional consequences of this interaction, we measured therapeutic endpoints after inhibition of NF-κB using JSH-23. We used a clonogenic assay to show that reproductive capacity was significantly reduced in JSH-23-treated malignant breast cells after IR (Fig. 1B). In addition, as we have previously shown that using 3D IrECM accurately predicted response between normal and malignant cells in vivo, we tested JSH-23 in this context. As shown, colony size and proliferation were dramatically reduced while apoptosis was increased in T4-2, but not S1, cells, consistent with the role of NF-κB in positively regulating β1-integrin post-IR in malignant, but not nonmalignant breast cells (Fig. 3 and Supplementary Fig. S3). To determine the broader applicability of our findings, we tested luminal-like (MCF7) and basal-like (MDA-MB-231) breast cancer cell lines for response to JSH-23 and IR. Interestingly, JSH-23 highly induced apoptosis and reduced proliferation in basal-like breast cancers T4-2 and MDA-MB-231 compared with luminal-like breast cancer MCF-7 cells (Figs. 3C–E and 4), indicating that basal-like breast cancers may be more responsive to β1-integrin/NF-κB–targeted therapies. Yamaguchi N and colleagues reported that basal-like breast cancer cell lines that exhibited higher activation of NF-κB than luminal subtypes are preferentially involved in proliferation (37). Together, these results are consistent with our previous findings that β1-integrin levels are much higher in basal compared with luminal cell lines, consistent with a higher dependence on integrin-related prosurvival signaling (8, 13, 28).

It has been well documented that the stress conditions induced by radiation can activate a cellular defense system, including NF-κB, the acute phase transcription factor that affects the decision of cell fate after IR. NF-κB mediates the survival response of many signals by inhibiting p53-dependent apoptosis and upregulating antiapoptotic members of the Bcl-2 family, and caspase inhibitors such as XIAP and FLIP (29, 38). In addition to antiapoptotic responses, NF-κB regulates expression of the stress-responsive genes associated with a prosurvival network (39, 40). Inhibition of NF-κB activity increases the intrinsic radiosensitivity in several (41, 42), but not all, human cancer cell lines (43). This apparent paradox indicates that IR-induced NF-κB subunits may cross-talk with other signaling elements and/or may have distinct tissue-specificity. To further investigate the relationship between β1-integrins and NF-κB, we provide evidence for components of the regulatory network showing that phosphorylation of T788/789 of the β1-integrin cytoplasmic tail were decreased by NF-κB inhibitors (JSH-23 and IMD-0354), along with total β1-integrins and the active components of the NF-κB p65/p50 subunits (Figs. 2C and E and Supplementary Fig. S1A).

This study also shows interactions of β1-integrin with NF-κB p65 and one of its heterodimeric partners α5-integrin. Protein–protein interactions can have positive implications for cancer prevention and therapy. The studies of Vassilev and colleagues (44, 45) showed in vivo proof-of-principle that inhibitors of protein–protein interactions can be efficacious anticancer drugs. NF-κB physically and/or functionally interacts with many proteins, including MEK, E2F1 transcription factor, and PML tumor suppressor, involved in the controlling of cell proliferation and survival (23, 46, 47). In the present study, we have shown that the interactions of β1-integrin with NF-κB p65 and α5-integrin were oppositely regulated in malignant breast cancer T4-2 and its counterpart radiosensitive nonmalignant breast epithelial S1 cells. As shown in Supplementary Fig. S4, β1-integrin/p65 protein complex significantly increased post-IR in S1, but not T4-2, cells, indicating that NF-κB may protect S1 cells against radiation damage via physical interaction with β1-integrin. Furthermore, IR-induced α5β1-integrin complex was much higher in T4-2 than S1 cells (Supplementary Fig. S4C), suggesting that the induction of β1-integrin interaction with α5-integrin promotes radioresistance of malignant breast cancer T4-2 cells.

Another important implication of the current results is the loop-like activation pathway of NF-κB/β1-integrin signaling in breast cancer radioresistance. It is well documented that NF-κB is activated by radiation (23, 29), and NF-κB is now shown to bind directly to the β1-integrin promoter, resulting in β1-integrin overexpression and tumor radioresistance. In addition, several previous studies have correlated NF-κB activation with integrin ligation. The functional role of integrin-induced NF-κB in cell survival was first shown by Scatena and colleagues (33). In Figs. 6A and B, we showed that IR-induced NF-κB p65/p50 expression and DNA binding activity were inhibited by β1-integrin function blocking monoclonal antibody AIIB2. This feed-forward loop-like β1-integrin–NF-κB–β1-integrin pathway activated post-IR may cause tumor resistance. A schematic presentation of the β1-integrin–NF-κB–β1-integrin loop in radioresistance is proposed in Fig. 6C. We speculate that activation of this pathway results in the failure of DNA-damaging anticancer modalities in breast cancers.

In summary, we report here a novel finding that β1-integrin is induced by exposure to radiation through NF-κB–mediated gene activation in 3D IrECM breast cancer cell culture and breast cancer xenografts. NF-κB–mediated β1-integrin overexpression is tightly associated with enhanced clonogenic survival and tumor repopulation. Our results
suggest that breast cancer therapy may be enhanced by targeting the NF-κB/β1-integrin pathway of radiation-resistant tumors.

Disclosure of Potential Conflicts of Interest

C.C. Park has ownership interest (including patents) in Oncosynergy. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: K.M. Ahmed, C.C. Park
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.M. Ahmed
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.M. Ahmed
Writing, review, and/or revision of the manuscript: K.M. Ahmed, C.C. Park

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NF-κB Regulates Radioresistance Mediated By β1-Integrin in Three-Dimensional Culture of Breast Cancer Cells

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