Published in final edited form as:

J Cell Physiol. 2009 September ; 220(3): 593–599. doi:10.1002/jcp.21765.

Inhibition of RelB by 1,25-Dihydroxyvitamin D₃ Promotes Sensitivity of Breast Cancer Cells to Radiation

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Abstract

Aberrant constitutive expression of the NF-κB c-Rel and RelA subunits in breast cancer cells was shown to promote their survival. Recently, we demonstrated that aggressive breast cancers constitutively express high levels of the RelB subunit, which promotes their more invasive phenotype via induction of the *BCL2* gene. As these cancers are frequently resistant to therapy, here we tested the hypothesis that RelB promotes their survival. High RelB expressing Hs578T and MDA-MB-231 breast cancer cells were more resistant to γ-radiation than MCF7 and ZR-75 cells, which express lower RelB levels. Knockdown of RelB in Hs578T led to decreased survival in response to γ-irradiation, while conversely ectopic expression of RelB in MCF7 cells protected these cells from radiation. Similar data were obtained upon treatment of Hs578T or MCF7 cells with the chemotherapeutic agent doxorubicin. High serum levels of 25-hydroxyvitamin D are associated with decreased breast cancer risk and mortality, although, the mechanism of its protective action has not been elucidated. Treatment of Hs578T and Her-2/neu-driven NF639 cells with 1,25-dihydroxyvitamin D₃ decreased *RelB/RELB* gene expression and levels of pro-survival targets Survivin, MnSOD and Bcl-2, while increasing their sensitivity to γ-irradiation. Thus, RelB, which promotes survival and a more highly invasive phenotype of breast cancer cells, is a target of 1,25-dihydroxyvitamin D₃, providing one mechanism for the observed protective role of 25-hydroxyvitamin D in patients with breast cancer.

Keywords

RelB; NF-κB; 1,25-dihydroxyvitamin D₃; Radiation; Breast cancer

NF-κB is a family of dimeric transcription factors that control genes which promote cell survival, proliferation and invasive phenotype (Ghosh et al., 1998; Rayet and Gelinas, 1999; Sonenshein, 1997). Mammals express five NF-κB members: c-Rel, RelB, RelA (also known as p65), p50 and p52. NF-κB factors bind as hetero- or homo-dimers, except for RelB which has a structurally different N-terminus and only binds as a heterodimer typically with either p50 or p52 (Siebenlist et al., 1994). NF-κB complexes differentially regulate gene expression possibly due to various interactions with other transcription factors or kinases and to different DNA binding specificities. For example, RelB is unable to induce

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transcription of the gene encoding the pro-survival factor Bcl-X_L, whereas RelA and c-Rel are potent activators (Jiang et al., 2002; Suhasini et al., 1997). In most untransformed cells, other than B lymphocytes, NF-κB complexes are sequestered in the cytoplasm bound to specific IκB inhibitory proteins. Several years ago, we and others demonstrated that over 90% of breast cancers aberrantly express constitutive nuclear c-Rel, p50, p52 or RelA NF-κB subunits, which promote resistance to chemotherapy (Cogswell et al., 2000; Nakshatri et al., 1997; Sovak et al., 1997; Wang et al., 1996). Little was known about a role for RelB in breast cancer. RelB was thought to function primarily in regulation of adaptive immune responses, specifically during differentiation of dendritic cells (Burkly et al., 1995; Clark et al., 1999). We first noted nuclear RelB in mouse mammary tumors induced by ectopic c-Rel expression (Romieu-Mourez et al., 2003) and then upon exposure to the carcinogen dimethyl[a]benzanthracene (Demicco et al., 2005). Subsequently, we observed an inverse correlation between constitutive RelB expression and estrogen receptor alpha (ERα) levels in breast cancer tissues and cell lines (Wang et al., 2007). Furthermore, RelB was shown to promote a more invasive phenotype via induction of Bcl-2.

Epidemiological data have demonstrated an association between high levels of serum 25-hydroxyvitamin D [25(OH)D] or vitamin D intake and a decreased risk for development of breast cancer (Bertone-Johnson et al., 2005; Shin et al., 2002). More recently, an inverse correlation between serum levels of 25(OH)D and breast cancer mortality has been observed (Garland et al., 2007). Vitamin D is a group of fat-soluble pro-hormones which includes two major forms, vitamin D₂ and vitamin D₃ (Hollis, 2008). Hydroxylation of these compounds on carbon 25 of the side chain and then carbon 1 of the A ring results in metabolic activation. In cells in culture, addition of the active 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] form has been implicated in cell cycle arrest, differentiation and apoptosis of breast cancer cells (Eisman et al., 1989; Lazzaro et al., 2000; Narvaez and Welsh, 2001). The mechanism of vitamin D action on breast cancer cells is complex. In ERα positive cells, the anti-proliferative effects of 1,25(OH)₂D₃ are thought to be at least in part mediated via repression of ERα levels and estrogen-mediated bioresponses (Swami et al., 2000). However, 1,25(OH)₂D₃ and its analogs are also potent inhibitors of proliferation in ERα negative cells (Elstner et al., 1995), thus indicating additional ERα independent mechanisms of action. Here, we tested the role of RelB in resistance of breast cancer cells to γ-irradiation and to the chemotherapeutic agent doxorubicin. RelB promoted survival of breast cancer cells to these two anti-cancer treatment modalities. Furthermore, 1,25(OH)₂D₃ inhibited the high RelB levels found in ERα negative breast cancers and thereby decreased their survival. Thus, inhibition of RelB by 1,25(OH)₂D₃ represents a novel mechanism to explain the association between high levels of serum 25-hydroxyvitamin D [25(OH)D] or vitamin D intake and a decreased risk of breast cancer.

Methods and Reagents

Cell culture and treatment conditions

ERα positive MCF7 and ZR-75 cells, and ERα negative Hs578T and MDA-MB-231 cells were maintained as described previously (Wang et al., 2007). The cell line NF639 (kindly provided by P. Leder, Harvard Medical School, Boston MA), which was derived from a mammary gland tumor in a MMTV-Her-2/neu transgenic mouse, was cultured as described previously (Pianetti et al., 2001). MCF7 cell lines containing pcDNA3 or pcDNA3-RelB and Hs578T cell lines containing human pREL_B-siRNA or control pREL_B sense (si-Control) were established as described (Wang et al., 2007) and grown in the presence of 400 μg/ml G418 (Sigma) or 1 μg/ml puromycin (Sigma), respectively. Where indicated, cells were treated with 10⁻⁵ M Doxorubicin (Sigma) or an equivalent volume of water. An initial dose response curve of the effects of Doxorubicin on survival indicated a significant difference between Hs578T-siRelB and Hs578T-Con cells was achieved at 10⁻⁵ M (not shown). For

1,25(OH)₂D₃ treatments, cells were exposed to 10⁻⁸ M 1,25(OH)₂D₃ (a generous gift from Dr. M. Uskokovic, Hoffmann LaRoche, Nutley, NJ) or an equivalent volume of ethanol. An initial dose response curve of the effects of 10⁻¹², 10⁻¹⁰, 10⁻⁸ and 10⁻⁶ M 1,25(OH)₂D₃ on RelB levels in Hs578T cells indicated effective inhibition was achieved at 10⁻⁸ M (not shown).

Transient Transfections

Cells cultures, at 90% confluency, were transfected with 10 µg of the RelB expression vector pMexNeo-RelB (a kind gift of Rodrigo Bravo, Bristol-Myers Squibb Co.) or the corresponding pMexNeo control empty vector (EV) using LipofectamineTM 2000 Reagent (Invitrogen, Carlsbad, CA), in Optimem serum-free medium, according to the manufacturer's protocol. After overnight transfection, Optimem was replaced with fresh complete medium and cells allowed to recover for 8 hours. The resulting NF639-EV and NF639-RelB cells were used immediately in experiments evaluating the effects of 1,25(OH)₂D₃ and γ-irradiation.

Immunoblot analysis and antibodies

Whole cell, nuclear and cytoplasmic extracts were prepared and immunoblotting performed as previously described (Wang et al., 2007). Antibodies against RelB NF-κB (sc-226) and the pro-survival factors Survivin (sc-10811) and Bcl-2 (sc-492) were purchased from Santa Cruz Biotechnology. Antibodies against Manganese superoxide dismutase (MnSOD; catalog no. 06-984) and β-actin were purchased from Upstate and Sigma, respectively. For quantitation of protein expression, blots from three separate experiments were scanned, subjected to densitometry and values normalized to β-actin. Average fold change ± standard deviation (SD) in protein levels relative to levels in control untreated cells (set at 1) are given.

Colony forming assay

For clonogenic cell survival studies, cells (200–500 cells/well) were seeded in 6-well plates in triplicate and after overnight incubation, subjected to the indicated doses of ionizing radiation in a 130 kV X-ray machine (Faxitron X-ray Corporation) at a dose rate of 89.7 cGy/min. For 1,25(OH)₂D₃ experiments, cells were either pre-treated with 1,25(OH)₂D₃ or a corresponding volume of vehicle ethanol for 24 h, and then irradiated. Cells were allowed to form colonies over a period of 5–10 days after irradiation. Colonies were counted if they contained 50 or more cells. Survival fraction (in log₁₀ scale) for the different radiation doses was calculated as a ratio of the number of colonies in each treatment condition over the total number of colonies in the corresponding untreated control. Values shown are averages of triplicate samples ± SD. Statistical significance between curves was determined using a paired Student's t-test and for a specific dose between the curves using a Student's t-test for samples with equal variance. P-values of <0.05 were considered statistically significant.

Cell viability assays

Cell viability was assessed with an APTlite 1step luminescence ATP detection assay system (Perkin Elmer), performed as recommended by the manufacturer. Briefly, samples (3000 or 8000 cells/100 µl for Hs578T or MCF7 cells, respectively) were plated in triplicate in 96-well plates and treated with the indicated dose of Doxorubicin for 24 h. An equal volume of APTlite 1step luminescence reagent was added to each well and luciferase activity measured in a Luminoskan Ascent 96-well luminometer. Viability (% Survival) was calculated as a ratio of the relative luminescence values for each treatment condition over those in corresponding untreated samples multiplied by 100. Values shown are averages of triplicate samples ± SD. The statistical significance was determined as described above.

For NF639 cell counting experiments, samples (4000 cells/well) were plated in triplicate in 6-well plates. After overnight incubation, cells were treated with the indicated dose of $1,25(\text{OH})_2\text{D}_3$ for 24 h, then exposed to 2 Grays of γ -irradiation and allowed to recover for 3 days. Cells numbers were counted in a Beckman Coulter Z2 Counter. Viability (% Survival) was calculated as a ratio of the number of cells in each treatment condition over the number of cells in the corresponding untreated control samples multiplied by 100. Values shown are averages of triplicate samples $\pm$ SD. The statistical significance was determined as described above.

RT-PCR

RNA was isolated using Trizol reagent (Invitrogen) and quantified by measuring A260. cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. RNA expression for *Vdr/VDR*, *RelB/RELB*, *Survivin/SURVIVIN*, *MnSOD/MNSOD*, *Bcl-2/BCL-2* and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh/GAPDH*), used as a loading control, was assessed by reverse transcription (RT)-PCR. PCR was performed in a thermal cycler as follows: for mouse *Bcl-2*, 30 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s; for human *MNSOD*, 24 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s; and for mouse *Vdr* and human *VDR*, 33 cycles at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 45 s. The following primer sets were used: for mouse *Bcl-2*, 5'-TATAAGCTGTCACAGAGGGGCTA-3' and 5'-CATGCTGGGGCCATATAGTT-3'; for mouse *Vdr*, 5'-CCAGGAATACCAAGGCTGAA-3' and 5'-TGGATAGGCGGTCCTGAATGGC-3'; for human *MNSOD*, 5'-GCACTAGCAGCATGTTGAGC-3' and 5'-CAACGCCTCCTGGTACTTCT-3'; and for human *VDR* 5'-ATGGCCATCTGCATCGTCTC-3' and 5'-CTTCCTCTTCGGCCTTTTCT-3'. The PCR conditions and primer sets used for mouse *RelB*, *MnSOD*, *Survivin* and *Gapdh*, and for human *RELB*, *SURVIVIN*, *BCL2* and *GAPDH* have been described previously (Mineva et al., 2007; Wang et al., 2007). For quantitation of RNA levels, gels from triplicate experiments were subjected to densitometry and normalized to *Gapdh/GAPDH*. Average fold change $\pm$ SD in mRNA levels relative to those in control untreated cells (set to 1) are presented.

Results

RelB regulates breast cancer cell resistance to ionizing radiation

Previously, we demonstrated an inverse correlation between ER α status and RelB levels in breast cancer cells (Wang et al., 2007). ER α positive ZR-75 and MCF7 expressed low RelB levels, while ER α negative Hs578T and MDA-MB-231 and ER α low NF639 breast cancer cells expressed high levels of RelB. To begin to assess the involvement of RelB in resistance of breast cancer cells to γ -irradiation, ZR-75, MCF7, Hs578T and MDA-MB-231 cell cultures were subjected to a gradient dose of γ -radiation followed by a clonogenic survival assay. As seen in Figure 1, high RelB-expressing Hs578T and MDA-MB-231 cells were more resistant to radiation-induced cell death compared to low RelB-expressing ZR-75 and MCF7 cells.

To test directly whether RelB is responsible for the observed resistance to radiation, we used paired stable cultures of Hs578T cells expressing siRNA *RELB* or control sense *RELB* RNA (Hs578T-si*RELB* vs Hs578T-Con) and MCF7 cells ectopically expressing RelB or empty vector control DNA (MCF7-RelB vs MCF7-EV cells), that had been prepared and characterized previously (Wang et al., 2007). Cells were subjected to a gradient dose of γ -irradiation followed by a clonogenic survival assay. Knockdown of RelB in Hs578T-si*RELB* cells led to increased killing following radiation compared to Hs578T-Con cells (Fig. 2A). Conversely, enforced RelB expression in MCF7-RelB cells led to increased survival upon

irradiation as compared to MCF7-EV cells (Fig. 2B). Thus, RelB promotes resistance of breast cancer cells to ionizing radiation.

RelB mediates breast cancer cell survival to doxorubicin

Doxorubicin (or Adriamycin) is one of the most important drugs available for the treatment of metastatic breast cancer and is highly effective in treatment of invasive HER-2 positive and ER α negative breast cancers (Paik et al., 1998; Paik et al., 2000; Paridaens et al., 2000). Thus, we next tested the ability of RelB to protect Hs578T and MCF7 breast cancer cells from death induced by treatment with doxorubicin. Cultures were treated with 10^{-5} M doxorubicin or with an equal volume of vehicle water as control for 24 h. Doxorubicin treatment induced a decrease in survival of both Hs578T-Con and Hs578T-si*REL*B cells (Fig. 3A), and the killing was further enhanced by knockdown of RelB. A moderate, statistically significant increase in the loss of cell viability by doxorubicin treatment was seen in the Hs578T-si*REL*B vs Hs578T-Con cells (Fig. 3A). Similarly, treatment of MCF7-EV with doxorubicin resulted in robust killing of these cells (with only 29.8% viable cells remaining) (Fig. 4A). RelB overexpression in MCF7 cells profoundly increased survival following doxorubicin treatment (Fig. 4A). We next asked whether doxorubicin effects RelB expression, and subjected whole cell extracts from treated cultures to immunoblot analysis for RelB (Fig. 3B). Unexpectedly we observed that doxorubicin treatment profoundly decreased RelB protein levels in the control Hs578T cells, such that they were comparable to those in untreated Hs578T-si*REL*B. Doxorubicin treatment of Hs578T-si*REL*B cells further reduced RelB expression to levels slightly lower than treated Hs578T-Con cells (Fig. 3B). Immunoblotting analysis demonstrated that while the ectopic RelB overexpression in MCF7-RelB was substantially reduced by doxorubicin treatment, consistent with a post-transcriptional site of regulation, the levels were substantially above those seen in the control MCF7-EV cells (Fig. 4B). Thus, RelB promotes survival of breast cancer cells to treatment with doxorubicin, which robustly represses the levels of this NF- κ B subunit.

1,25(OH) $_2$ D $_3$ inhibits RelB expression in breast cancer cells and increases sensitivity to γ -irradiation

Recently, RelB has been implicated in sensitivity of prostate cancer cells to treatment with 1,25(OH) $_2$ D $_3$ (Xu et al., 2007). Thus, the effects of 1,25(OH) $_2$ D $_3$ on RelB levels were assessed in Hs578T breast cancer cells, and in a second line NF639, which also expresses high levels of RelB (Wang et al., 2007). Cultures were treated with 10^{-8} M 1,25(OH) $_2$ D $_3$ or vehicle ethanol, as control, and total RNA and whole cell extracts isolated. Treatment of Hs578T cells with 1,25(OH) $_2$ D $_3$ resulted in a robust decrease in *REL*B mRNA (Fig. 5A) and in a commensurate reduction in RelB protein levels (Fig. 5B). Similarly, treatment of NF639 cells with 1,25(OH) $_2$ D $_3$ decreased *Rel*B RNA and RelB protein levels (Figs. 6A and 6B). When the effects of 1,25(OH) $_2$ D $_3$ treatment on RelB pro-survival target genes was examined, a substantial decrease in mRNA and protein expression of *Survivin*, *MnSOD*, and *Bcl2* was noted in the NF639 cells (Figs. 6A and 6B). Similarly, *MNSOD*, *SURVIVIN* and *BCL2* gene expression in Hs578T cells was reduced by 1,25(OH) $_2$ D $_3$ treatment (Figs. 5A and 5B). The presence of 1,25(OH) $_2$ D $_3$ receptor RNA was confirmed in these lines, as well as in the MDA-MB-231, MCF7, and ZR-75 cells (Supplementary Fig. 1), as expected. Thus 1,25(OH) $_2$ D $_3$ treatment inhibits functional RelB levels in breast cancer cells.

We next tested whether treatment of these breast cancer cells with 1,25(OH) $_2$ D $_3$ enhances their sensitivity to γ -irradiation. Hs578T cells were pre-treated with 10^{-8} M 1,25(OH) $_2$ D $_3$ for 24 h, exposed to a gradient dose of γ -radiation and subjected to a clonogenic survival assay. As seen in Figure 5C, pre-treatment with 1,25(OH) $_2$ D $_3$ resulted in a dose-dependent increase in the sensitivity of Hs578T cells to γ -irradiation. Statistically significant differences in sensitivity were seen at doses of 3, 4, 5, and 6 Gray. Similarly, NF639 cells

displayed an increased sensitivity to γ -irradiation following pre-treatment with 1,25(OH) $_2$ D $_3$ (Fig. 6C). Thus, 1,25(OH) $_2$ D $_3$ treatment enhances sensitivity to radiation treatment.

To confirm that the increased sensitivity to γ -irradiation mediated by 1,25(OH) $_2$ D $_3$ was due to inhibition of RelB, NF639 cells were transiently transfected with the pMexNeo-RelB vector (NF639-RelB) or the corresponding control EV (NF639-EV) DNA. Immunoblot analysis of whole cell extracts confirmed that NF639-RelB cells expressed higher levels of RelB as compared to NF639-EV cells (Fig. 7A). The NF639-RelB and NF639-EV cells were either left untreated, pre-treated with 10^{-8} M 1,25(OH) $_2$ D $_3$ for 24 h, exposed to 2 Grays of γ -irradiation, or a combination of both and allowed to recover for 3 days and RelB protein expression and cell survival assessed. Immunoblot analysis of whole cell extracts isolated following these treatments revealed that exposure to 1,25(OH) $_2$ D $_3$ alone led to substantial decreases in RelB levels in NF639-RelB vs NF639-EV cells (Fig. 7B). In the NF639-EV cells, the effects of irradiation alone were more modest and the combination appeared similar to 1,25(OH) $_2$ D $_3$ alone. In the NF639-RelB, irradiation did not result in a decrease in RelB levels and RelB levels in the cells given the combination of irradiation and 1,25(OH) $_2$ D $_3$ were maintained at almost twice that of the 1,25(OH) $_2$ D $_3$ alone (Fig. 7B). The effects of the treatments with 1,25(OH) $_2$ D $_3$ and irradiation on survival of the NF639-RelB and NF639-EV cultures were then assessed. NF639-RelB cells were found to be significantly less sensitive to treatment with a combination of 1,25(OH) $_2$ D $_3$ and γ -irradiation or to γ -irradiation alone as compared to control NF639-EV cells, consistent with retention of higher RelB levels during these treatments (Fig. 7C). Furthermore, treatment with 1,25(OH) $_2$ D $_3$ alone decreased cell survival equally in NF639-RelB and NF639-EV cells, consistent with a lack of difference in RelB levels seen above (Fig. 7B). Thus, ectopic RelB impairs the killing by a combination of 1,25(OH) $_2$ D $_3$ and radiation treatments in these breast cancer cells.

Discussion

Here, RelB was shown to promote survival of breast cancer cells to γ -irradiation and doxorubicin, two commonly used therapeutic treatments. Furthermore, exposure to 1,25(OH) $_2$ D $_3$ was found to repress *REL*B gene expression and thereby to increase sensitivity to irradiation. The ability of RelB to promote survival can be attributed to its ability to induce the anti-apoptotic proteins Survivin, MnSOD, and Bcl-2. In particular, we showed that the reduction in levels of RelB by 1,25(OH) $_2$ D $_3$ treatment, sensitized the ER α negative Hs578T cells and Her-2/neu-driven NF639 cells, which are ER α low, to γ -irradiation treatment. An adjuvant effect of 1,25(OH) $_2$ D $_3$ and analogs on doxorubicin and irradiation has been reported for ER α positive breast cancer cells (Chaudhry et al., 2001). To our knowledge, this is the first report of similar effects of 1,25(OH) $_2$ D $_3$ in the response of ER α negative or Her-2/neu-driven breast cancer cells to irradiation. Recent work suggests a similar scenario involving RelB and 1,25(OH) $_2$ D $_3$ occurs in prostate cancer. Induction of MnSOD by RelB played an important radioprotective role in aggressive PC-3 prostate cancer cells (Josson et al., 2006). RelB was identified as a potential prognostic marker in prostate cancer, as nuclear RelB levels statistically correlated with high Gleason score (Lessard et al., 2005). More recently, Xu and co-workers demonstrated a drop in RelB levels and enhanced radiosensitivity of aggressive prostate cancer cells following treatment with 1,25(OH) $_2$ D $_3$ (Xu et al., 2007). Overall, these results suggest a significant role for repression of RelB in the observed correlation of serum 25(OH)D levels and decreased risk of breast cancer.

Previously, we demonstrated that RelB promotes a more invasive phenotype of breast cancer cells (Wang et al., 2007). Consistent with our findings, Kattan and coworkers recently demonstrated that suppression of MnSOD in breast cancer cells results in a decrease in

colony forming ability and invasive properties (Kattan et al., 2007). Bcl-2 and Survivin have been associated in breast cancer patients with a low probability of response to chemotherapy and poor prognosis, respectively (Bonetti et al., 1998; Span et al., 2004). Reduced apoptotic indices and poorer survival rates were noted in breast cancer cases that were positive for both Survivin and Bcl-2 (Tanaka et al., 2000). These findings suggest Survivin and Bcl-2 may cooperate to promote breast cancer. Of note, in our current study, RelB expression led to concurrent activation of MnSOD, Bcl-2 and Survivin and resistance to chemotherapy and radiation. Preliminary data from microarray analysis (data not shown) suggest that RelB regulates the expression of a larger set of genes involved in control of survival. However, further studies are necessary to elucidate their specific roles in RelB-mediated breast cancer cell survival and response to therapy.

Doxorubicin is a common anticancer treatment, which has been found to function via several cytostatic and cytotoxic mechanisms of action. These include (1) intercalation into DNA, which results in inhibition of macromolecular biosynthesis; (2) free radical formation; and (3) inhibition of topoisomerase II, which leads to induction of DNA damage (Gewirtz, 1999). While early studies showed that doxorubicin, and other topoisomerase inhibitors increase nuclear NF- κ B levels (Bottero et al., 2001; Wang et al., 1996), these have more recently been shown to be functionally inactive by Ho and coworkers (2005). Specifically, classical NF- κ B p50/p65 binding complexes induced by doxorubicin treatment in breast cancer cells are inactive due to lack of post-transcriptional modifications, resulting in decreased NF- κ B target gene expression (Ho et al., 2005). In the present study, doxorubicin was shown to act as a potent RelB inhibitor. The decrease in classical NF- κ B activity may contribute to the reduced RelB levels, as its promoter has two functional NF- κ B elements (Bren et al., 2001; Wang and Sonenshein, 2005). Consistent with the ability of doxorubicin to inhibit RelB levels, no additive effects of 1,25(OH) $_2$ D $_3$ and doxorubicin on RelB levels or survival were observed in NF639 or Hs578T cells (data not shown). Doxorubicin is a standard treatment for advanced breast cancers and has been found particularly effective against aggressive Her-2 positive and ER α negative subtypes (Gianni et al., 2005; Kuerer et al., 1999; Mortimer et al., 1985; Paik et al., 1998; Paik et al., 2000). Importantly, our previous studies demonstrated that these aggressive cancers express high levels of RelB. Overall, these findings suggest that doxorubicin is an effective treatment against these breast cancers, in part, because of its ability to reduce their high RelB levels, and therefore survival of these cells.

In summary, our data demonstrate that the RelB NF- κ B subunit promotes survival of breast cancer cells and that 1,25(OH) $_2$ D $_3$ reduces RelB levels and therefore survival. Together these observations provide one mechanism to explain the recently reported association between decreased breast cancer risk and mortality and serum levels of 25(OH)D (Bertone-Johnson et al., 2005; Garland et al., 2007). Our studies suggest that further analysis of the potential beneficial role of 1,25(OH) $_2$ D $_3$ as an adjuvant in radiation treatment of breast cancers with high RelB levels is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Contract grant sponsor: NIH;

Contract grant numbers: R01 CA129129 and P01 ES11624.

Contract grant sponsor: AICR (with funds from the Derx Foundation);

Contract grant number: 07A-151.

GES is supported by grants R01 CA129129 and P01 ES11624. NDM is supported by a fellowship from the AICR (with funds from the Derx Foundation). We thank Tai Cheng Chen for his providing reagents.

Literature Cited

- Bertone-Johnson ER, Chen WY, Holick MF, Hollis BW, Colditz GA, Willett WC, Hankinson SE. Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. *Cancer Epidemiol Biomarkers Prev.* 2005; 14(8):1991–1997. [PubMed: 16103450]
- Bonetti A, Zaninelli M, Leone R, Cetto GL, Pelosi G, Biolo S, Menghi A, Manfrin E, Bonetti F, Piubello Q. bcl-2 but not p53 expression is associated with resistance to chemotherapy in advanced breast cancer. *Clin Cancer Res.* 1998; 4(10):2331–2336. [PubMed: 9796962]
- Bottero V, Busuttill V, Loubat A, Magne N, Fischel JL, Milano G, Peyron JF. Activation of nuclear factor kappaB through the IKK complex by the topoisomerase poisons SN38 and doxorubicin: a brake to apoptosis in HeLa human carcinoma cells. *Cancer Res.* 2001; 61(21):7785–7791. [PubMed: 11691793]
- Bren GD, Solan NJ, Miyoshi H, Pennington KN, Pobst LJ, Paya CV. Transcription of the RelB gene is regulated by NF-kappaB. *Oncogene.* 2001; 20(53):7722–7733. [PubMed: 11753650]
- Burkly L, Hession C, Ogata L, Reilly C, Marconi LA, Olson D, Tizard R, Cate R, Lo D. Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature.* 1995; 373(6514):531–536. [PubMed: 7845467]
- Chaudhry M, Sundaram S, Gennings C, Carter H, Gewirtz DA. The vitamin D3 analog, ILX-23-7553, enhances the response to adriamycin and irradiation in MCF-7 breast tumor cells. *Cancer Chemother Pharmacol.* 2001; 47(5):429–436. [PubMed: 11391859]
- Clark GJ, Gunningham S, Troy A, Vuckovic S, Hart DN. Expression of the RelB transcription factor correlates with the activation of human dendritic cells. *Immunology.* 1999; 98(2):189–196. [PubMed: 10540217]
- Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin AS Jr. Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. *Oncogene.* 2000; 19(9):1123–1131. [PubMed: 10713699]
- Demicco EG, Kavanagh KT, Romieu-Mourez R, Wang X, Shin SR, Landesman-Bollag E, Seldin DC, Sonenshein GE. RelB/p52 NF-kappaB complexes rescue an early delay in mammary gland development in transgenic mice with targeted superrepressor IkappaB-alpha expression and promote carcinogenesis of the mammary gland. *Mol Cell Biol.* 2005; 25(22):10136–10147. [PubMed: 16260626]
- Eisman JA, Sutherland RL, McMenemy ML, Fragonas JC, Musgrove EA, Pang GY. Effects of 1,25-dihydroxyvitamin D3 on cell-cycle kinetics of T 47D human breast cancer cells. *J Cell Physiol.* 1989; 138(3):611–616. [PubMed: 2925799]
- Elstner E, Linker-Israeli M, Said J, Umiel T, de Vos S, Shintaku IP, Heber D, Binderup L, Uskokovic M, Koeffler HP. 20-epi-vitamin D3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. *Cancer Res.* 1995; 55(13):2822–2830. [PubMed: 7796409]
- Garland CF, Gorham ED, Mohr SB, Grant WB, Giovannucci EL, Lipkin M, Newmark H, Holick MF, Garland FC. Vitamin D and prevention of breast cancer: pooled analysis. *J Steroid Biochem Mol Biol.* 2007; 103(3–5):708–711. [PubMed: 17368188]
- Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochemical pharmacology.* 1999; 57(7):727–741. [PubMed: 10075079]
- Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol.* 1998; 16:225–260. [PubMed: 9597130]
- Gianni L, Zambetti M, Clark K, Baker J, Cronin M, Wu J, Mariani G, Rodriguez J, Carcangiu M, Watson D, Valagussa P, Rouzier R, Symmans WF, Ross JS, Hortobagyi GN, Pusztai L, Shak S. Gene expression profiles in paraffin-embedded core biopsy tissue predict response to

- chemotherapy in women with locally advanced breast cancer. *J Clin Oncol*. 2005; 23(29):7265–7277. [PubMed: 16145055]
- Ho WC, Dickson KM, Barker PA. Nuclear factor-kappaB induced by doxorubicin is deficient in phosphorylation and acetylation and represses nuclear factor-kappaB-dependent transcription in cancer cells. *Cancer Res*. 2005; 65(10):4273–4281. [PubMed: 15899819]
- Hollis BW. Measuring 25-hydroxyvitamin D in a clinical environment: challenges and needs. *Am J Clin Nutr*. 2008; 88(2):507S–510S. [PubMed: 18689391]
- Jiang HY, Petrovas C, Sonenshein GE. RelB-p50 NF-kappa B complexes are selectively induced by cytomegalovirus immediate-early protein 1: differential regulation of Bcl-x(L) promoter activity by NF-kappa B family members. *Journal of virology*. 2002; 76(11):5737–5747. [PubMed: 11992002]
- Josson S, Xu Y, Fang F, Dhar SK, St Clair DK, St Clair WH. RelB regulates manganese superoxide dismutase gene and resistance to ionizing radiation of prostate cancer cells. *Oncogene*. 2006; 25(10):1554–1559. [PubMed: 16261162]
- Kattan Z, Minig V, Leroy P, Dauca M, Becuwe P. Role of manganese superoxide dismutase on growth and invasive properties of human estrogen-independent breast cancer cells. *Breast Cancer Res Treat*. 2007
- Kuerer HM, Newman LA, Smith TL, Ames FC, Hunt KK, Dhingra K, Theriault RL, Singh G, Binkley SM, Sneige N, Buchholz TA, Ross MI, McNeese MD, Buzdar AU, Hortobagyi GN, Singletary SE. Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy. *J Clin Oncol*. 1999; 17(2):460–469. [PubMed: 10080586]
- Lazzaro G, Agadir A, Qing W, Poria M, Mehta RR, Moriarty RM, Das Gupta TK, Zhang XK, Mehta RG. Induction of differentiation by 1alpha-hydroxyvitamin D(5) in T47D human breast cancer cells and its interaction with vitamin D receptors. *Eur J Cancer*. 2000; 36(6):780–786. [PubMed: 10762752]
- Lessard L, Begin LR, Gleave ME, Mes-Masson AM, Saad F. Nuclear localisation of nuclear factor-kappaB transcription factors in prostate cancer: an immunohistochemical study. *Br J Cancer*. 2005; 93(9):1019–1023. [PubMed: 16205698]
- Mineva ND, Rothstein TL, Meyers JA, Lerner A, Sonenshein GE. CD40 ligand-mediated activation of the de novo RelB NF-kappaB synthesis pathway in transformed B cells promotes rescue from apoptosis. *J Biol Chem*. 2007; 282(24):17475–17485. [PubMed: 17446175]
- Mortimer J, Flournoy N, Livingston RB, Stephens RL. Aggressive adriamycin-containing regimen (PM-FAC) in estrogen receptor-negative disseminated breast cancer. Results of a Southwest Oncology Group trial. *Cancer*. 1985; 56(10):2376–2380. [PubMed: 3899347]
- Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ Jr, Sledge GW Jr. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol*. 1997; 17(7):3629–3639. [PubMed: 9199297]
- Narvaez CJ, Welsh J. Role of mitochondria and caspases in vitamin D-mediated apoptosis of MCF-7 breast cancer cells. *J Biol Chem*. 2001; 276(12):9101–9107. [PubMed: 11053435]
- Paik S, Bryant J, Park C, Fisher B, Tan-Chiu E, Hyams D, Fisher ER, Lippman ME, Wickerham DL, Wolmark N. erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. *Journal of the National Cancer Institute*. 1998; 90(18):1361–1370. [PubMed: 9747867]
- Paik S, Bryant J, Tan-Chiu E, Yothers G, Park C, Wickerham DL, Wolmark N. HER2 and choice of adjuvant chemotherapy for invasive breast cancer: National Surgical Adjuvant Breast and Bowel Project Protocol B-15. *Journal of the National Cancer Institute*. 2000; 92(24):1991–1998. [PubMed: 11121461]
- Paridaens R, Biganzoli L, Bruning P, Klijn JG, Gamucci T, Houston S, Coleman R, Schachter J, Van Vreckem A, Sylvester R, Awada A, Wildiers J, Piccart M. Paclitaxel versus doxorubicin as first-line single-agent chemotherapy for metastatic breast cancer: a European Organization for Research and Treatment of Cancer Randomized Study with cross-over. *J Clin Oncol*. 2000; 18(4):724–733. [PubMed: 10673513]

- Pianetti S, Arsura M, Romieu-Mourez R, Coffey RJ, Sonenshein GE. Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. *Oncogene*. 2001; 20(11): 1287–1299. [PubMed: 11313873]
- Rayet B, Gelinas C. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene*. 1999; 18(49): 6938–6947. [PubMed: 10602468]
- Romieu-Mourez R, Kim DW, Shin SM, Demicco EG, Landesman-Bollag E, Seldin DC, Cardiff RD, Sonenshein GE. Mouse mammary tumor virus c-rel transgenic mice develop mammary tumors. *Mol Cell Biol*. 2003; 23(16):5738–5754. [PubMed: 12897145]
- Shin MH, Holmes MD, Hankinson SE, Wu K, Colditz GA, Willett WC. Intake of dairy products, calcium, and vitamin d and risk of breast cancer. *Journal of the National Cancer Institute*. 2002; 94(17):1301–1311. [PubMed: 12208895]
- Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol*. 1994; 10:405–455. [PubMed: 7888182]
- Sonenshein GE. Rel/NF-kappa B transcription factors and the control of apoptosis. *Semin Cancer Biol*. 1997; 8(2):113–119. [PubMed: 9299589]
- Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM, Sonenshein GE. Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. *J Clin Invest*. 1997; 100(12):2952–2960. [PubMed: 9399940]
- Span PN, Sweep FC, Wiegerinck ET, Tjan-Heijnen VC, Manders P, Beex LV, de Kok JB. Survivin is an independent prognostic marker for risk stratification of breast cancer patients. *Clin Chem*. 2004; 50(11):1986–1993. [PubMed: 15364883]
- Suhasini M, Reddy CD, Reddy EP, DiDonato JA, Pilz RB. cAMP-induced NF-kappaB (p50/relB) binding to a c-myc intronic enhancer correlates with c-myc up-regulation and inhibition of erythroleukemia cell differentiation. *Oncogene*. 1997; 15(15):1859–1870. [PubMed: 9362453]
- Swami S, Krishnan AV, Feldman D. 1alpha,25-Dihydroxyvitamin D3 down-regulates estrogen receptor abundance and suppresses estrogen actions in MCF-7 human breast cancer cells. *Clin Cancer Res*. 2000; 6(8):3371–3379. [PubMed: 10955825]
- Tanaka K, Iwamoto S, Gon G, Nohara T, Iwamoto M, Tanigawa N. Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. *Clin Cancer Res*. 2000; 6(1):127–134. [PubMed: 10656440]
- Wang CY, Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science*. 1996; 274(5288):784–787. [PubMed: 8864119]
- Wang X, Belguise K, Kersual N, Kirsch KH, Mineva ND, Galtier F, Chalbos D, Sonenshein GE. Oestrogen signalling inhibits invasive phenotype by repressing RelB and its target BCL2. *Nat Cell Biol*. 2007; 9(4):470–478. [PubMed: 17369819]
- Wang X, Sonenshein GE. Induction of the RelB NF-kappaB subunit by the cytomegalovirus IE1 protein is mediated via Jun kinase and c-Jun/Fra-2 AP-1 complexes. *Journal of virology*. 2005; 79(1):95–105. [PubMed: 15596805]
- Xu Y, Fang F, St Clair DK, Josson S, Sompol P, Spasojevic I, St Clair WH. Suppression of RelB-mediated manganese superoxide dismutase expression reveals a primary mechanism for radiosensitization effect of 1alpha,25-dihydroxyvitamin D(3) in prostate cancer cells. *Mol Cancer Ther*. 2007; 6(7):2048–2056. [PubMed: 17604335]

Figure 1.

High RelB-expressing breast cancer cells are less susceptible to γ -radiation than low-RelB-expressing cells. Hs578T, MDA-MB-231, MCF7 and ZR-75 breast cells were either left untreated or subjected to the indicated doses of ionizing radiation. Treatments were done in triplicate. A clonogenic survival assay was performed to assess resistance to radiation. The survival fraction (log10 scale) over the different radiation doses for each cell line was plotted. Values shown are averages of triplicate samples $\pm$ SD.

Figure 2.

RelB promotes resistance of breast cancer cells to ionizing radiation. A: Stable Hs578T transfectants expressing either RelB siRNA (Hs578T-siRELB) or a control sense RelB oligonucleotide (Hs578T-Con) were prepared as described previously (Wang et al., 2007). Hs578T-Con and Hs578T-siRELB cells were either left untreated or subjected to the indicated doses of ionizing radiation and assessed for viability as described in Figure 1. Comparison of the two curves using a paired Student's t-test indicated a statistically significant difference, $p\text{-value} = 8.1 \times 10^{-7}$. *, point of statistically significant difference ($p\text{-value} < 0.05$) between the two curves. B: Stable MCF7 transfectants expressing either RelB (MCF7-RelB) or a control empty vector (MCF7-EV) were prepared as described previously (Wang et al., 2007). MCF7-EV and MCF7-RelB cells were either left untreated or subjected to the indicated doses of ionizing radiation and assessed for viability, as above. Comparison of the two curves using a paired Student's t-test indicated a statistically significant difference, $p\text{-value} = 1.2 \times 10^{-6}$. *, point of statistically significant difference ($p\text{-value} < 0.05$) between the two curves.

Figure 3.

Knockdown of RelB increases sensitivity of breast cancer cells to treatment with doxorubicin. Hs578T-Con and Hs578T-siRELB cells were treated with media containing 10^{-5} M doxorubicin (DOXO) or a corresponding volume of water for 24 h. A: Cell viability was assessed with an APTlite 1step luminescence ATP detection assay. The percent survival relative to untreated controls, set to 100%, is given. Values shown are averages of triplicate samples $\pm$ SD. A Student's t-test revealed significant differences in survival between untreated and doxorubicin treated Control cells or siRELB expressing cells (p-value <0.05). *, The effects of doxorubicin treatment on Control vs siRELB cells were statistically different as assessed by a Student's t-test with equal variance (p-value = 0.009). B: Alternatively, whole cell extracts were prepared and samples (20 μ g) subjected to immunoblot analysis for RelB and β -actin as a loading control.

Figure 4.

Ectopic expression of RelB increases resistance of breast cancer cells to doxorubicin treatment. MCF7-EV and MCF7-RelB cells were treated with media containing 10^{-5} M doxorubicin or a corresponding volume of water for 24 h. A: MCF7-EV and MCF7-RelB cells were treated and analyzed for cell viability as described in Figure 3A. A Student's t-test revealed significant differences in survival between untreated and doxorubicin treated Control cells or RelB expressing cells (p -value < 0.05). *, The effects of doxorubicin treatment on Control vs RelB cells were statistically different as assessed by a Student's t-test with equal variance (p -value = 9.3×10^{-6}). B: Alternatively, samples of whole cell extracts (20 μ g) were subjected to immunoblot analysis for RelB and β -actin.

Figure 5.

1,25(OH)₂D₃ treatment of Hs578T cells leads to inhibition of RelB and increased sensitivity to γ-irradiation. Hs578T cells were treated with 10⁻⁸ M 1,25(OH)₂D₃ or vehicle control ethanol for 24 h. A: RNA was isolated and analyzed by RT-PCR for levels of *RELB*, *SURVIVIN*, *MNSOD*, *BCL2* and *GAPDH*, as a control for equal loading. B: Whole cell extracts were isolated and samples (20 μg) subjected to immunoblot analysis for RelB, Survivin, Bcl-2, MnSOD and β-actin as a loading control. C: Hs578T cells were treated with 10⁻⁸ M 1,25(OH)₂D₃ or vehicle control ethanol for 24 h. Following 1,25(OH)₂D₃ treatment, cells were subjected to the indicated doses of ionizing radiation. Treatments were done in triplicate. A clonogenic survival assay was performed. Survival fraction in log10 scale over the different radiation doses was plotted. Values shown are averages of triplicate samples ± SD. Comparison of the two curves using a paired Student's t-test indicated a statistically significant difference, p-value = 9.2 e⁻⁰⁵. *, point of statistically significant difference (p-value <0.05) between the two curves.

Figure 6.

1,25(OH)₂D₃ treatment of NF639 cells leads to inhibition of RelB and increased sensitivity to γ -irradiation. NF639 cells were treated with 10⁻⁸ M 1,25(OH)₂D₃ or vehicle control ethanol for 24 h. A: RNA was isolated and analyzed by RT-PCR for levels of *RelB*, *Survivin*, *MnSOD*, *Bcl-2* and *Gapdh*, as a control for equal loading. B: Whole cell extracts were isolated and samples (20 μ g) subjected to immunoblot analysis for RelB, Survivin, MnSOD, Bcl-2 and β -actin as a loading control. C: NF639 cells were treated with 10⁻⁸ M 1,25(OH)₂D₃ or vehicle control ethanol for 24 h. Following 1,25(OH)₂D₃ treatment, cells were subjected to the indicated doses of ionizing radiation. Treatments were done in triplicate. A clonogenic survival assay was performed. Survival fraction in log10 scale over the different radiation doses was plotted. Values shown are averages of triplicate samples $\pm$ SD. Comparison of the two curves using a paired Student's t-test indicated statistical significance, p-value = 8.1 e⁻⁵. *, point of statistically significant difference (p-value <0.05) between the two curves.

Figure 7. RelB overexpression decreases 1,25(OH)₂D₃ mediated sensitization of breast cancer cells to γ-irradiation. NF639 cells were transiently transfected with a pMexNeo-RelB vector (NF639-RelB) or the corresponding control EV (NF639-EV) DNA. A: Whole cell extracts were isolated, following overnight transfection and 8h recovery in fresh media, and samples (20 μg) subjected to immunoblot analysis for RelB and β-actin as a loading control. B and C: Following recovery, NF639-RelB and NF639-EV cells, seeded in 6-well plates and allowed to settle overnight, were treated with 10⁻⁸ M 1,25(OH)₂D₃ or vehicle control ethanol for 24 h. Cells were then subjected to 2 Grays of γ-irradiation and allowed to recover for 3 days. B: Whole cell extracts were isolated and samples (20 μg) subjected to immunoblot analysis for RelB and β-actin as a loading control. C: Cell numbers in each treatment condition were counted on a Beckman Coulter Z2 Counter and presented as a percentage (% Survival) of the corresponding untreated sample (set to 100). Values shown are averages of triplicate samples ± SD. *, significant difference in survival between NF639-RelB and NF639-EV cells treated with γ-irradiation alone or a combination of 1,25(OH)₂D₃

and γ -irradiation as assessed by a Student's t-test for samples with equal variance (p-value = 0.002 and 0.004, respectively).