Acquisition of Stable Inducible Up-Regulation of Nuclear Factor-κB by Tumor Necrosis Factor Exposure Confers Increased Radiation Resistance without Increased Transformation in Breast Cancer Cells

Steve Braunstein,1 Silvia C. Formenti,2 and Robert J. Schneider1

Departments of 1Microbiology and 2Radiation Oncology, New York University School of Medicine, New York, New York

Abstract
High-grade breast cancers are better adapted to hypoxia and more resistant to chemotherapy and radiotherapy. Constitutive activation of the transcription factor nuclear factor-κB (NF-κB) increases in breast tumors and in breast cancer cell lines, where it promotes chemoradiation resistance, in part by activation of antiapoptotic genes. The role for up-regulation of NF-κB in breast cancer progression is less clear. Here, we first show that whereas the constitutive activity of NF-κB is incrementally elevated from immortalized breast epithelial to frank transformed invasive ductal breast cancer cell lines (3-fold, ±0.1-fold, P < 0.05), inflammatory cytokine-inducible activity is further increased (up to 9-fold, ±0.9-fold, P < 0.05). We then show that inhibition of NF-κB activity selectively sensitizes transformed but not immortalized cells to killing by ionizing radiation or low levels of tumor necrosis factor (TNF) by up to 10-fold (±1-fold, P < 0.05) but has little effect on hypoxia-mediated cell death. Prolonged cultivation of immortalized and partially transformed cells in TNF selected for cells displaying stable constitutive and strongly inducible overexpression of NF-κB even in the absence of TNF. Stable acquisition of increased NF-κB activity conferred resistance to ionizing radiation or inflammatory cytokines, which was dependent on elevated NF-κB activity, but had no effect on transformation potential measured by in vitro and in vivo parameters. Thus, TNF and possibly other inflammatory cytokines in the tumor-stroma matrix likely select for breast cancer cells that stably overexpress NF-κB, leading to greater cancer cell survival. Greater cell survival despite increased genomic injury may permit increased acquisition of malignant genetic alterations as well as resistance to chemoradiation therapy. (Mol Cancer Res 2008;6(1):78–88)

Introduction
The increased constitutive activation of nuclear factor-κB (NF-κB) has been observed in several human solid tumors, including breast, pancreatic, colon, and melanoma, as well as leukemias and lymphomas (refs. 1-4, reviewed in ref. 5). NF-κB up-regulation is thought to result largely from increased activity of its activating kinase, IκB kinase β (1). In breast cancers, constitutive activation of NF-κB has been associated with development of hormone-independent estrogen receptor-α-negative (ER-α) cancers (4, 6, 7) as well as progression of a select subset of aggressive hormone-dependent ER+ breast cancers (8). In both cases, increased disease progression is thought to result from NF-κB stimulation of cyclin D genes and increased cell cycle progression, from increased activation of antiapoptotic genes bcl-2 and bcl-xL, that are NF-κB dependent, from increased expression of vascular endothelial growth factor and consequent tumor angiogenesis and activation of the proto-oncogene c-myc, and from increased metalloproteinase gene expression and the resulting remodeling of the extracellular matrix (9-13). There is also some evidence that constitutive up-regulation of NF-κB activity is predictive of chemoradiation and radiation treatment response (reviewed in ref. 14).

It remains unclear why certain breast cancers overexpress NF-κB in association with disease progression, and the reasons are likely multifaceted. For instance, NF-κB activation is associated with the hypoxic tumor microenvironment (15). NF-κB activation has also been associated with progression from ER- to ER+ breast cancer as well as ErbB-2 (Her2/neu) overexpression (16, 17). Clearly, elevated levels of NF-κB are also associated with chemoradiation and radiation therapy resistance, but this is itself a surrogate for resistance to tumor cell killing likely acquired by stresses in the tumor microenvironment (reviewed in ref. 18). In this regard, studies have shown that pharmacologic and enzyme inhibitors of NF-κB promote radiosensitivity in tissue culture and mouse models of breast and other cancers (4, 19-25).

In this report, we have explored the association between the state of constitutive and inducible overexpression of NF-κB, breast cancer cell transformation, ER, Her2/neu status, and the activity of proinflammatory cytokines and resistance of tumor...
cells to ionizing radiation (IR), hypoxia, and inflammatory cytokines. We show that tumor necrosis factor (TNF) selects for stable acquisition of strongly increased inducible NF-κB activity, primarily in immortalized breast epithelial cells and, to a lesser extent, in breast cancer cells. Acquisition of NF-κB activity is shown to be singularly responsible for resistance to killing by inflammatory cytokines and IR but not to hypoxia. Our results suggest that stable acquisition of increased NF-κB activity does not directly alter the state of breast cancer cell transformation but by promoting cancer cell survival, and in the presence of proinflammatory genomic injury, it might permit increased acquisition of malignant genetic alterations.

**Results**

**Inducible Activation of NF-κB Increases Strongly with Transformation in Breast Cancer Cell Lines**

The basal (constitutive) and inducible levels of NF-κB activity were assessed in a panel of breast cancer cell lines. MCF10A are immortalized breast epithelial cells that grow poorly at low density and cannot form tumors in nude mice (Fig. 1A and B; refs. 26, 27). UACC-893 cells were derived from a grade 3 ER/PR+ p53+/Her2/neu+++ tumor that are anchorage dependent, grow poorly at low density, and only form tumors in nude mice when implanted with the basement membrane Matrigel (Fig. 1A and B). Thus, MCF10A cells represent nontransformed (immortalized) breast epithelial cells and UACC-893 cells are moderately transformed cells based on standard in vitro and in vivo laboratory growth criteria despite their original high grade of derivation and Her2/neu overexpression. HCC70 and BT474 cells were derived from grade 3 ER/PR+, Her2/neu+++ tumors and display increased transformation based on these same criteria. As anticipated from previous reports, compared with immortalized MCF10A cells, these cell lines displayed 3- to 5-fold increased levels of constitutive NF-κB activity, measured by transfection of a NF-κB–dependent luciferase reporter plasmid normalized for equal transfection efficiencies (Fig. 1C). Moreover, the level of NF-κB activity inducible by inflammatory cytokine TNF was found to increase strikingly compared with immortalized MCF10A cells, 5-fold in UACC-893 cells, 7-fold in HCC70 cells, and 9-fold in BT474 cells (Fig. 1C). A similar trend of lower but still significant magnitude was observed for interleukin (IL)-1β stimulation of NF-κB activity. At the levels of cytokines used, there was no evidence for significant cell killing over a 24-h period, but above these values, MCF10A cell death started to become apparent (data not shown). Cotransfection of an expression vector for the super-repressor inhibitor of NF-κB, known as IκB-SR, blocked inflammatory cytokine activation of NF-κB. IR induction of NF-κB activity also showed greater effect in transformed BT474 compared with immortalized MCF10A breast cancer cells (Fig. 1D), although the magnitude was significantly reduced compared with cytokine activation. Significant induction of NF-κB activity was only observed in transformed BT474 cells and plateaued by 8 Gy irradiation. As shown in other reports (23, 28, 29), although IR activates NF-κB, it is generally weaker and with slower kinetics (on the order of hours) compared with rapid and more robust activation by inflammatory cytokines.

The slower and weaker activation of NF-κB by IR was found to be a direct effect of IR and not a result of IR induction of NF-κB activating secreted factors. Conditioned medium was obtained from MCF10A and BT474 cells at either 0.5 or 4 h following 8 Gy irradiation. Conditioned medium was used to replace the medium on nonirradiated matched cell lines that were previously transfected with a NF-κB–responsive luciferase reporter. Luciferase activity was assessed 8 h later (Fig. 1E). There was no significant induction of NF-κB activity by the conditioned medium from IR-treated cells (P < 0.01, ANOVA). Moreover, an ELISA-based multicytokine array analysis (Pannomics) of the conditioned medium failed to detect significantly elevated levels of TNF, IL-1β, or other cytokines that could activate NF-κB (data not shown). Thus, immediate early induction of NF-κB activity is a direct effect on nonautocrine radiation-sensitive intrinsic signaling pathways.

**NF-κB Inhibition Selectively Enhances Transformed Cell Killing by Radiation or TNF but not Hypoxia**

To facilitate analysis of NF-κB inhibition in longer duration studies than can be achieved by transient plasmid expression of NF-κB inhibitor IκB-SR, we used prostaglandin A1 (PGA1), shown previously to specifically block NF-κB activation by irreversibly inhibiting its activating kinase IκB kinase β (30). All breast cancer cell lines were equally sensitive to inhibition of TNF-mediated NF-κB activation at 50 μmol/L PGA1 as shown by inhibition of a NF-κB–dependent luciferase reporter (Fig. 2A), with no discernible effects on cell viability over a period of 24 h (data not shown), as reported previously (30). PGA1 at 50 μmol/L was therefore used to block NF-κB activation and the effect of cytotoxic agents on cell viability was determined for the panel of breast cancer cell lines. To enable direct comparison of cell lines across the different cytotoxic agents, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used, which is a measure of short-term cell viability in vitro. The clonogenic cell growth assay is a measure of long-term cell viability and is not amenable to determination of cell viability at the time of cell stress, such as IR, hypoxia, or exposure to inflammatory cytokines. Inhibition of NF-κB had no effect on cell killing by IR for immortalized MCF10A cells, moderately sensitized UACC-893 cells, and more strongly sensitized HCC70 and BT474 cells, which express a higher level of NF-κB activity (Fig. 2B). Inhibition of NF-κB activity rendered both highly transformed cell lines to a similar level of sensitivity to killing by a cytotoxic level of TNF (50 ng/mL/24 h; Fig. 2C). There was no effect of PGA1 itself on cell viability in the absence of the cytotoxic action of TNF (Fig. 2C). Equally surprising, inhibition of NF-κB activity did not increase the sensitivity of cell lines to killing by prolonged hypoxia (0.5% O2, 24 h) regardless of the level of transformation (Fig. 2D). In general, cells become resistant to hypoxia with increased transformation and hypoxia can activate NF-κB (30). These data therefore suggest that acquisition of increased NF-κB activity may uniformly protect cells against killing mediated by inflammatory cytokines or IR but not hypoxia, which can also up-regulate NF-κB activity, independent of the state of cell transformation, hormone receptor, or Her2/neu expression levels. Studies were therefore carried out to test this hypothesis.
**Cells Stably Acquire Strongly Increased, Cytokine-Inducible Levels of NF-κB in Response to Killing by TNF**

Given the observed correlation between elevated NF-κB activity and protection against TNF, we asked whether increased NF-κB activity is a characteristic that can be stably selected in sensitive cell populations to provide protection against killing by proinflammatory cytokines. We therefore first determined whether overexpression of NF-κB in sensitive cell populations is sufficient to provide resistance to killing by TNF. Our previous studies showed that expression of RelA, the transcription activating member of NF-κB, is sufficient to confer on cells increased NF-κB activity (31).

**FIGURE 1.** Strong elevation in inducible compared with constitutive NF-κB activity with increased transformation in breast cancer cell lines. 

A. Cell growth was assessed by the ability to proliferate at low density by seeding 10,000 per 10-cm dish and scoring the number of proliferating cells after 10 d. Results were done twice in triplicate. 

B. Cell lines were tested for tumor formation by injection of 2 × 10⁶ cells into 6-week-old female NCR-nu mice (eight per group) without or with Matrigel as indicated. Data are presented as average tumor size during growth. 

C. Inflammatory cytokine induction of NF-κB activity. Established cell lines of increasing transformation corresponding to immortalized breast epithelial cells (MCF10A), stage II grade 3 invasive ductal carcinoma (UACC-893), stage IIIA grade 3 (HCC70), and stage IV grade 3 (BT474) were propagated under identical conditions and transfected with a NF-κB–activated luciferase reporter construct. Cells were either mock treated or treated with TNF or IL-1β as shown and NF-κB transcriptional activity was measured as the number of absolute light units. Columns, mean of at least three independent experiments; bars, SE.

D. IR induction of NF-κB activity. MCF10 and BT474 cells were transfected with a NF-κB luciferase reporter construct and cotransfected GFP reporter to normalize transfection efficiencies and then treated with the indicated doses of IR at 1 Gy/min, and NF-κB–activated luciferase activity was measured 8 h later, a time when activity reached a plateau. Note the two log decrease in luciferase activity scale compared with activation by TNF or IL-1β.

E. Conditioned medium from IR-treated MCF10A or BT474 cells does not activate NF-κB. Cells were treated with IR and conditioned medium was collected 30 min or 4 h later, used to replace medium on untreated corresponding cells that had been transfected with a NF-κB–dependent luciferase expression reporter. Columns, mean NF-κB (luciferase) activity derived from at least three independent experiments; bars, SE. All results shown are typical of at least three independent experiments and achieve P < 0.01 (paired Student’s t test) compared with untreated controls.
The panel of breast cancer cell lines was transiently transfected with an expression plasmid for RelA, normalized for transfection efficiency, and then exposed to either 10 or 50 ng/mL of TNF for 2 days and the immediate effect on viability was determined by MTT assay. Data are presented as the percent increase in survival compared with untreated control cells. There was little increased survival for breast cancer cell lines BT474 and HCC70 that are already resistant to TNF and express high levels of NF-κB activity. However, immortalized MCF10A cells, and to a lesser extent transformed UACC-893 cells, which naturally express lower levels of NF-κB activity, showed significant gains in survival with RelA overexpression (Fig. 3A). As a control for NF-κB activity, transfected RelA was shown to strongly increase IL-1β activation of...
a NF-κB–dependent luciferase reporter in both MCF10A and BT474 cells (Fig. 3B).

Cell lines were therefore cultured in the presence of 50 ng/mL TNF for 4 weeks (Fig. 3C). This concentration of TNF over a period of 1 week kills over 95% of MCF10A cells, 60% of UACC-893 cells, and ~10% of HCC70 and BT474 cells (data not shown). Clonal populations of surviving cells were then pooled to normalize representation of a mixed cell population and passaged for five generations in the absence of TNF selection. Cells were then examined for constitutive and inducible levels of NF-κB activity by transfection with a NF-κB–dependent luciferase reporter plasmid. Data are presented as the fold increase in NF-κB activity of the TNF-selected cell lines compared with the unselected parental cells. All data were normalized to a cotransfected green fluorescent protein (GFP) reporter plasmid to eliminate differences in transfection efficiency. Although there was only a slight increase in the basal activity levels of NF-κB in the TNF-selected cell lines compared with parental cell lines (data not shown), there was a profound increase in IL-1β or TNF-inducible NF-κB activity in immortalized MCF10A cells (9- and 11-fold, respectively) and less so in transformed cells, with UACC-893 cells showing the largest gain (3-fold) and a negligible increase in HCC70 and BT474 cells (Fig. 3D). Thus, prolonged exposure of breast cancer cell lines that normally display low levels of NF-κB activity to a cytotoxic concentration of TNF can select for cells with a stably increased level of proinflammatory cytokine-inducible NF-κB activity.

**TNF-Selected Cell Lines Acquire Resistance to Lethal Levels of TNF and IR**

Studies were carried out to determine whether the acquisition of strongly inducible levels of NF-κB activity in cells surviving TNF selection confers increased activation by, and

**FIGURE 3.** TNF selects for stable, strongly increased, inducible NF-κB activity in immortalized and moderately transformed breast cancer cell lines. **A.** Transient expression of RelA promotes greatest survival benefit in immortalized and weakly transformed cells exposed to TNF. Breast cancer cell lines were transfected with an expression plasmid for RelA and a cotransfected GFP reporter to normalize transfection efficiencies and a NF-κB luciferase reporter and then exposed for 24 h to 10 or 50 ng/mL of TNF and viability was determined by MTT assay. Results are presented as the percent increase in survival compared with cells not transfected with RelA. Columns, mean of at least three independent experiments; bars, SE. **B.** Cell lines described above were analyzed for NF-κB activity without and with activation by 0.1 μg/mL IL-1β. Results from three independent experiments are presented as the fold increase of RelA-expressing cells compared with control cells. **C.** Schema for selection of TNF-resistant clones. Cell lines were exposed to 50 ng/mL TNF for 4 wk, and resistant clones representing <5% of the original cells were pooled and passaged for five doublings in the absence of TNF selection before studies. **D.** TNF-selected immortalized MCF10A cells acquire strongest inducible increase in NF-κB activity. Following TNF selection, pooled clones from cell lines were transfected with a NF-κB luciferase reporter plasmid and a cotransfected GFP reporter to normalize transfection efficiencies and treated for 2 h with 10 pg/mL IL-1β or 10 ng/mL TNF, and luciferase activity was determined, measured as the number of absolute light units from at least three independent experiments. Results are presented as the fold increase of TNF-selected compared with unselected cell lines. \( P < 0.01 \), paired Student’s t test.
resistance to, killing by IR in addition to inflammatory cytokines. Compared with highly transformed BT474 cells, MCF10A cells and, to a lesser extent, UACC-893 cells acquired strongly increased IR-inducible NF-κB activity, similar in trend but reduced in effect compared with the effects of inflammatory cytokine induction (Fig. 5A).

To compare resistance to high levels of TNF with that of IR, a short-duration survival analysis was conducted using the MTT assay. Analysis of long-term survival to IR was conducted by clonogenic assay. The viability of the panel of breast cancer cell lines to increasing levels of TNF was compared before TNF selection (Fig. 4B) and after TNF selection (Fig. 4C). As a control, studies also examined TNF selected after inhibition of NF-κB activity by PGA1 (Fig. 4D). In the absence of TNF selection, MCF10A cells, which displayed the lowest endogenous levels of NF-κB activity, showed the highest sensitivity to killing by low levels of TNF over a period of 2 days. UACC-893 cells, which displayed higher levels of NF-κB activity, were more resistant, whereas BT474 and HCC70 cell lines were largely resistant, commensurate with their innate high levels of NF-κB activity. Following TNF selection, as described above, immortalized MCF10A cells, which showed the largest gain in IR-inducible NF-κB activity, also showed the greatest increase in resistance to killing following irradiation. UACC-893 cells showed a significant but lesser gain in resistance to killing (Fig. 4C), commensurate with their increase in IR-inducible NF-κB activity. For instance, before TNF selection, 80% of MCF10A cells were killed by 100 ng/mL TNF over 2-day exposure, whereas after selection ~80% of cells now survived. Inhibition of NF-κB activity with PGA1 significantly but not fully restored sensitivity to TNF-mediated killing to all of the selected cell lines (Fig. 4C), indicating that up-regulated NF-κB activity acquired during TNF selection is largely responsible for resistance to cell death. Similar data were obtained for single fraction irradiation of cell lines as well (Fig. 5B-D). For example, immortalized MCF10A cells, and to a lesser extent UACC-893 cells, were sensitive to killing by a single fraction of 8 Gy of IR before TNF selection (Fig. 5B) but acquired ~90% resistance following TNF selection (Fig. 5C), consistent with their relative increased IR-inducible NF-κB activity. The resistance to IR was largely dependent on the newly gained increase in NF-κB activity through TNF selection, as shown by restoration of cell killing with NF-κB inhibition (Fig. 5D).

The viability of unselected and TNF-selected immortalized MCF10A cells, and transformed UACC-893 cells following IR exposure, was investigated using a 14-day clonogenic cell survival assay and full dose-response curves were generated. Following mock treatment or treatment with increasing levels of a single fraction of IR, cells were dilution plated and surviving cell clones were scored after 2 weeks of growth. TNF-selected UACC-893 cells increased in their long-term resistance to 8 Gy of IR by 10-fold (Fig. 6A), and MCF10A cells increased by ~8-fold (Fig. 6B), compared with non–TNF-selected controls. The dose enhancement ratio, the extrapolation number (n) of intrinsically resistant cells, and the multitarget equation variable (D0) were calculated for the two cell lines without and with TNF selection by linear quadratic analysis, with the assumption of exponential dose responsiveness to IR, according to Iliakis and Okayasu (32) and Thames and Suit (Table 1; ref. 33). Only surviving fractions ≤0.1 were fitted (34). Data achieved a 95% confidence interval. The results show n values of 6.91 for control versus 1.56 for TNF-selected MCF10A cells, a difference of 4.5-fold, and 2.67 for control versus 1.49 for TNF-selected UACC-893 cells, respectively (Table 1). Similarly, D0 values of the final slope of the dose-response curves showed a decrease of about half for TNF-selected MCF10A and UACC-893 cells. Dose enhancement ratio calculated at 0.1 showed surviving fraction ratios of 0.8 for unselected compared with TNF-selected MCF10A cells and 0.62 for unselected versus TNF-selected UACC-893 cells. Thus, there is a clear sharpening of increased radiation resistance with TNF selection. There was no change in the
survival of TNF-selected HCC70 and BT474 cells as expected, consistent with the results of Fig. 4 (data not shown). These data show that TNF can select for stable acquisition of NF-κB activity in breast cancer cells, which is largely responsible for providing resistance to killing by IR.

**Stable Acquisition of Increased NF-κB Activity Does Not Alter the State of Breast Cancer Cell Line Transformation**

Whether increased levels of NF-κB activity are themselves transforming or play no direct role in transformation has not been resolved, particularly in breast cancer cells, and may to a large extent depend on the context of expression, the type of cells involved, and the developmental stage of the tissue during NF-κB expression (5).

We therefore investigated whether the acquisition of increased NF-κB activity in immortalized and transformed breast cancer cell lines was capable of increasing any measurable in vitro and in vivo parameters of transformation. None of the cell lines was significantly changed in doubling times after TNF selection. Mean doubling times for MCF10A cells were 26 to 28 h regardless of TNF selection, 24 h for UACC-893 cells, 20 h for HCC70 cells, and 18 h for BT474 cells (data not shown). The ability of cell lines to form colonies at low density in soft agar was also unchanged over the course of 10 days of growth despite TNF selection or addition of estrogen for ER+ HCC70 and BT474 cells (Fig. 6C). Of note, immortalized MCF10A cells cannot typically form tumors, and there was no increased ability to do so for their counterpart TNF-selected cells even when implanted subcutaneously within Matrigel (Fig. 6D). When implanted within Matrigel, it was apparent that TNF selection and acquisition of increased NF-κB activity did not increase their tumor formation ability (Fig. 6D). These data therefore indicate that stable acquisition of strong inducible NF-κB activation by selective growth of nontransformed or transformed breast cancer cells in the presence of TNF does not result in the acquisition of a greater transformed phenotype as measured by four independent in vitro and in vivo parameters.

**Discussion**

TNF is a key factor in the progression of early neoplastic lesions to development of breast cancer. It paradoxically can promote proliferation and apoptotic destruction of tumor cells as well as induce tumor angiogenesis, the latter through activation of metalloproteinases and increased vascular endothelial growth factor production (reviewed in ref. 35). At least one important decision factor in the TNF survival versus cell death paradox is the activation of NF-κB by TNF, clearly a prosurvival and possibly pro-proliferative transcription factor in human and animal models of different cancers (5). In benign low-grade breast cancers, TNF levels in the tumor-stromal microenvironment and the increased presence of TNF receptors I and II on tumor cells are associated with increasing...
malignancy (36-38). Whether NF-κB functions as a tumor promoter of inflammation-linked cancer in premalignant cells, as suggested from several animal studies (39), or more indirectly as a prosurvival factor that permits acquisition of transforming mutations in premalignant cells (40) is not resolved (5). The role of TNF up-regulation of NF-κB is no doubt more complex than just these two functions, as it has also been shown to also promote breast cancer cell invasiveness, probably by activation of metalloproteinases (37).

In this study, we investigated the association of elevated NF-κB activity with the state of breast cancer cell transformation, as assessed using in vitro and in vivo transformation studies. We showed that premalignant-derived breast cancer cell lines contain low levels of both constitutive (basal) and inducible NF-κB activity but with transformation gain a moderate increase in basal NF-κB activity and a striking increase in inflammatory cytokine or IR-inducible NF-κB activity. It is unclear as to the molecular mechanism of enhanced NF-κB activity in the more highly transformed cell lines. The high level of NF-κB activity in highly transformed breast cancer cells was found to serve as a prosurvival factor for these cells when exposed to high levels of proinflammatory cytokines, such as TNF or IR, but not during hypoxia. Because premalignant but not highly transformed cells were sensitive to killing by TNF, these data suggested that TNF might select for protective acquisition of high levels of NF-κB activity typically found in more transformed cells. Selection of premalignant cells with high levels of TNF for an extended period showed that the small surviving fraction of cells stably gained slightly higher basal levels, and much greater inducible levels of NF-κB activity, even after prolonged passaging in the absence of TNF. The stable acquisition of high NF-κB activity was found to promote survival of premalignant cells to cytotoxic insults but did not confer increased proliferation or transformation. These data therefore indicate that TNF can select for strongly and stably elevated levels of NF-κB in premalignant breast cancer cells, which confers a direct survival advantage.

Although it is also possible that elevated levels of NF-κB could confer enhanced cancer cell proliferation and transformation, given the established effects of elevated NF-κB activity on increased expression of vascular endothelial growth factor,

TABLE 1. Extrapolation Number (n), Dose Enhancement Ratio, and Multitarget Variable (D0) for IR Response of Unselected and TNF-Selected Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>D0</th>
<th>n</th>
<th>DER</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF10A</td>
<td>None</td>
<td>1.29 ± 0.10</td>
<td>6.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF selected</td>
<td>2.48 ± 0.15</td>
<td>1.57</td>
<td>0.80</td>
</tr>
<tr>
<td>UACC-893</td>
<td>None</td>
<td>1.63 ± 0.10</td>
<td>2.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF selected</td>
<td>3.46 ± 0.20</td>
<td>1.50</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Abbreviation: DER, dose enhancement ratio.

FIGURE 6. TNF-selected cell lines show increased radioresistant survival by clonogenic assay but not increased transformation. MCF10A (A) and UACC-893 (B) cell lines derived from TNF-selected pooled clones were compared with unselected lines in resistance to killing by increasing doses of IR up to 8 Gy at a dose rate of 1 Gy/min. Clonogenic assay was done as described in Materials and Methods for 2 wk following exposure. Results represent the average of at least three independent experiments and are presented as the surviving fraction of cells to the log10. C. TNF-resistant cell lines show no statistically significant increase in anchorage-independent growth in soft agar. Cell lines before and after TNF selection were tested for growth in 0.3% soft agar supplemented with RPMI 1640. The number of colonies was quantified at days 6 and 10 and averaged from three independent experiments done in duplicate. D. TNF-resistant cell lines show no increased ability to form tumors in nude mice. Cell lines before and after TNF selection were tested for tumor formation in nude mice by subcutaneous injection of 2 × 10^6 cells into 6-wk-old female NCR-nu mice (eight mice per group). Because MCF10A and UACC-893 cells do not typically form tumors in mice, a duplicate set of studies was done in which they were injected into a transplanted bed of Matrigel to support tumor growth. Data are presented as the tumor burden (number of tumors formed × the average tumor diameter in mm) at 4 wk from studies done in duplicate.
Her2/neu receptor, metalloproteinases, production of cytokines, invasiveness and decreased ER levels, among other activities, a direct causative role in increased transformation potential of breast cancer cells is less likely (5). In this regard, studies have found an association between elevated NF-κB levels in early-stage breast cancers and loss of estrogen and ER requirements for tumor cell growth, which itself promotes more aggressive disease (refs. 4, 8, reviewed in ref. 18). Other studies also found that inhibition of NF-κB itself had little effect on the proliferation of breast cancer cells in culture in the absence of additional mitogenic signals (17) or other activities, such as up-regulation of vascular endothelial growth factor and increased tumor angiogenesis (41). Most recently, it was shown that cytokine activation of NF-κB kinase IκB kinase α represses Maspin gene expression and inhibitor of metastatic progression (42). These data suggest a key role for cytokine-mediated NF-κB activation in metastogenicity, but not transformation per se, consistent with the results presented here. NF-κB overexpression therefore likely plays an important role in protecting tumor cells from induction of cell death during proliferation by specific mitogenic and chemotoxic agents and in metastatic events following frank transformation.

The relevance of TNF-selected, stably elevated levels of NF-κB in premalignant breast cancer cells derives from the importance of tumor-associated macrophages in promoting the progression of early premalignant breast cancers to malignant disease (reviewed in ref. 35). Tumor-associated macrophages produce a variety of proinflammatory cytokines, such as vascular endothelial growth factor, TNF, and IL-8, at the site of tumor formation (35). Elevated levels of tumor-associated macrophages and TNF in early breast cancer lesions are strongly associated with more aggressive progression to metastatic disease, higher metastatic potential, shorter time to recurrence after treatment, and higher levels of tumor vascularization (35). Our results suggest that TNF may select for premalignant breast cancer cells that survive by stably elevating strongly inducible levels of NF-κB, affording these cells a strong survival advantage against proinflammatory cytokines in the tumor microenvironment (and secondarily to cytotoxic antitumor agents such as IR), which indirectly promotes increased transformation through resistance to cell death and the ability to accumulate transforming mutations.

Luciferase Assays

Cells were transfected using Fugene reagent and a plasmid containing multimerized NF-κB-binding sites adjacent to a minimal promoter and firefly luciferase reporter (pNF-κB-Luc, Stratagene). NF-κB overexpression was achieved with RelA(p65) and inhibition with IκB-SR (super-repressor) constructs (gift of M. Karin, University of California, San Diego, CA). At 24 h after transfection, although cells were <70% confluent, they were treated for 2 h with IL-1β or TNF or with IR in the presence or absence of PGA1 at 0 to 50 μmol/L (30), which was a gift of G. Santoro (University of Rome, Rome, Italy). Equal amounts of protein lysates were assessed by measuring absolute light units for activity using a luminometer and the Bright-Glo luciferase system (Promega). Mock untreated control cells were used for standardization and normalization of results. Transfection efficiencies were assessed using an hrGFP construct (Stratagene), with quantification by FACSCalibur (New York University Cancer Institute). Studies are presented as the mean of at least three independent experiments with SEs shown.

Hypoxia Treatments

Hypoxic culture conditions (0.5% O2) were achieved in a custom-designed hypoxia incubator by a continuous infusion of a preanalyzed gas mixture (95% N2, 5% CO2; Remington Bioinstruments). All experiments were done with exponentially growing cells plated at ~40% cell density and then made hypoxic 18 to 24 h later when they were 70% confluent, similar to normoxic cells. Hypoxic medium was obtained by pre-equilibration for 6 h in the hypoxia chamber. Normoxic cells used for comparison were grown and treated under atmospheric oxygen in parallel.

Materials and Methods

Cell Culture

Human breast cancer cell lines were obtained from the American Type Culture Collection. Cell lines consist of MCF10A immortalized breast epithelial cells, UACC-893 cells derived from a TMM2 primary stage invasive ductal carcinoma (grade 3, Her2/neu+, p53+, ER/PR+), HCC70 cells derived from a TMM3A primary invasive ductal carcinoma (grade 3, Her2/neu+, p53+, ER/PR+), and BT474 cells derived from a stage IV invasive ductal carcinoma (grade 3, Her2/neu+, p53+, ER/PR+). Cells were grown under the guidelines of the American Type Culture Collection in their recommended medium. Cells were transfected with DNA plasmids using Fugene reagent as described by the manufacturer (Roche).

Materials and Methods

Cell Culture

Human breast cancer cell lines were obtained from the American Type Culture Collection. Cell lines consist of MCF10A immortalized breast epithelial cells, UACC-893 cells derived from a TMM2 primary stage invasive ductal carcinoma (grade 3, Her2/neu+, p53+, ER/PR+), HCC70 cells derived from a TMM3A primary invasive ductal carcinoma (grade 3, Her2/neu+, p53+, ER/PR+), and BT474 cells derived from a stage IV invasive ductal carcinoma (grade 3, Her2/neu+, p53+, ER/PR+). Cells were grown under the guidelines of the American Type Culture Collection in their recommended medium. Cells were transfected with DNA plasmids using Fugene reagent as described by the manufacturer (Roche).

[35S]Methionine Incorporation Assay

Cells were labeled with 50 μCi/mL [35S]methionine (EasyTag Express Protein Labeling Mix, Dupont/NEN) in methionine-free DMEM for 1 h and lysates were prepared as described previously (26). Specific activity of methionine incorporation was determined by trichloroacetic acid precipitation onto GF/C filters and liquid scintillation counting. Labeling of hypoxic samples was done within the hypoxia chamber with peroxide medium.

Growth in Soft Agar

A mix of 0.3% soft agar medium was prepared by combining an equal volume of autoclave sterilized low melting point agarose with RPMI 1640, held at 40°C, and an equal number of treated or mock-treated cells was added (200 per well in a six-well plate) and plated in triplicate. Cells were photographed at 6 and 10 days and colonies were counted.

IR Treatment

Cells were irradiated with a cobalt-60 irradiator at a dose rate of 1 Gy/min at room temperature for up to 10 Gy. Mock-treated cells were handled identically except for irradiation.

Clonogenic Cell Survival Assay

Cells were mock treated or treated with IR as above, harvested, and plated at equal cell numbers from 1,000 to 100,000.
in triplicate and maintained under culture conditions for up to 14 days to allow visual colony formation of surviving foci. Plating efficiencies for the surviving fraction of cells were determined from macroscopic colonies stained with 0.5% crystal violet solution in 90% methanol to fix cell foci.

**Cell Proliferation Rates**

Exponentially growing cells were harvested from plates with trypsin and replated in multwell plates for up to 12 days at different starting densities. Following treatments, cell proliferation was quantified by MTT assay as described by the manufacturer (Promega).

**Cell Growth at Low Density**

Cells were seeded at 10,000 per 10-cm plate and allowed to grow over 10 days. Cell numbers were determined by counting equal quadrants in triplicate plates.

**TNF Selection of Cells**

Cells were plated at 70% confluency and maintained in standard tissue culture medium with addition of human TNF at 50 ng/ml for 4 weeks. Medium and TNF were replaced every 3 days for 4 weeks. Cell death (~95% for weakly transformed cell lines MCF10A and UACC-893) resulted in development of clonogenic foci, which were pooled and maintained in tissue culture without TNF selection for five passages before studies.

**Subcutaneous Xenotransplant Tumor Model**

Tumors were established in 6-week-old female NCR-nu CD1 nude mice (eight mice per arm) by subcutaneous injection of \(2 \times 10^6\) tumor cells in the mid-dorsal region in both flanks. When necessary, exogenous estrogen was supplied by implantation of 60-day estrogen release pellets containing 0.72 mg \(\beta\)-estradiol (Innovative Research of America). Tumor volumes were measured by short and long tumor axis with calipers (length \(\times\) width\(^2\)) \(\times 0.5\). Tumor diameters represent the average of the short and long tumor axes.

**Statistical Analysis**

A two-tailed Student’s \(t\) test was done for statistical analysis of data, with \(P < 0.05\) taken as the level of significance.

References


Downloaded from mcr.aacrjournals.org on December 18, 2021. © 2008 American Association for Cancer Research.


Molecular Cancer Research

Acquisition of Stable Inducible Up-Regulation of Nuclear Factor-κB by Tumor Necrosis Factor Exposure Confers Increased Radiation Resistance without Increased Transformation in Breast Cancer Cells

Steve Braunstein, Silvia C. Formenti and Robert J. Schneider


Updated version
Access the most recent version of this article at:
http://mcr.aacrjournals.org/content/6/1/78

Cited articles
This article cites 42 articles, 20 of which you can access for free at:
http://mcr.aacrjournals.org/content/6/1/78.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://mcr.aacrjournals.org/content/6/1/78.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/6/1/78. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.