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Overexpression of the Myeloma Associated Oncogene Fibroblast Growth Factor Receptor 3 (FGFR3) Confers Dexamethasone Resistance.

Short Title: FGFR3 overexpression confers Dex resistance in MM cells.

Scientific Section: Neoplasia

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ABSTRACT

Translocations involving the immunoglobulin heavy chain switch region and fibroblast growth factor receptor 3 (FGFR3) are identified in 10-15% of myeloma patients. In previous work we overexpressed FGFR3 or the constitutively active FGFR3-TD mutant in an interleukin-6 (IL-6)-dependent murine myeloma cell line, B9. FGFR3 enhanced IL-6 responsiveness increased phosphorylation of STAT3 and up-regulated Bcl-x_L. Since Bcl-x_L was up-regulated, we have now tested FGFR3-expressing B9 cells for chemotherapy sensitivity. FGFR3 expression did not alter sensitivity to Melphalan or Doxorubicin. In contrast, B9 cells overexpressing FGFR3 were resistant to treatment with Dexamethasone, a phenomenon successfully reversed using a Bcl-x_L anti-sense oligonucleotide. This data thus demonstrates that overexpression of FGFR3 in B9 cells confers resistance to Dexamethasone, but not to anthracyclines or alkylating agents, at least in part through the upregulation of Bcl-x_L. This finding has potential implications for the use of chemotherapy in t(4;14)-positive myeloma.

INTRODUCTION

Translocations involving the immunoglobulin heavy (IgH) chain switch region, and a recurring series of translocation partners, are now known to occur in the majority of multiple myeloma (MM) patients¹⁻⁵. One of these, a karyotypically silent t(4;14)(p16;q32.3) translocation, is identified in approximately 15% of MM patients⁴⁻⁹. This translocation has been associated with aberrant FGFR3 expression and subsequent FGFR3 mutations that render the generated protein constitutively active^{7,9}. Activating mutations of FGFR3 have been reported in 10% of myeloma patients who are t(4;14)-positive and approximately 2% in myeloma patients overall¹⁰.

We transduced the interleukin-6 (IL-6)-dependent murine myeloma cell line, B9, with retroviruses expressing functional wild-type or constitutively active FGFR3¹¹. Overexpression of both wild-type and mutant FGFR3 genes resulted in decreased apoptosis, an increased proliferative response to IL-6, and at very high levels of FGFR3 expression, IL-6 independence¹¹. B9 clones ectopically expressing FGFR3 also displayed increased phosphorylation of STAT3 and higher levels of Bcl-x_L expression when compared to controls. Furthermore, levels of both proteins remained consistently high even after cytokine withdrawal¹¹. Overexpression of Bcl-x_L has been suggested as a marker of chemoresistant disease¹². Furthermore, Bcl-x_L expression in malignant plasma cells has been shown to strongly correlate with a decreased response rate to chemotherapeutic agents¹², and has been demonstrated to play an important role in protection from apoptosis by growth factor starvation of myeloma cells¹³. Therefore, we

hypothesized that the upregulation of Bcl-x_L by FGFR3 may also be sufficient to confer resistance to common chemotherapy agents in malignant plasma cells.

MATERIALS AND METHODS

Cell lines, Retroviral vectors and Tissue culture conditions

The B9 cell lines, viruses, and tissue culture conditions used in these experiments have been previously described¹¹. For IL-6 rescue experiments, cell lines were washed three times with 1x PBS to remove any residual IL-6, and cultured in IMDM supplemented with 5% FCS and penicillin-streptomycin (Gibco, Gaithersburg, MA). Twenty-four hours post-IL-6 withdrawal the media was supplemented with 2% IL-6-conditioned media. The human myeloma cell lines, (OPM1, OPM2, LP1, UTMC2, KHM11, KMS11 and H929) were grown in IMDM supplemented with 5% FCS and penicillin-streptomycin.

Antisense preparation and Treatment

The 2'-O-methoxyethyl chimeric phosphorothioate oligodeoxynucleotides used in this study were synthesized by Sigma (Milwaukee, WI). The Bcl-x_L antisense, 5'-CTGGATCCAAGGCTCTAGGT-3', and a control nonsense sequence oligo, 5'-CTGGATCCAAGGATCGAGGT-3', not recognizing any known gene, have been previously reported and characterized¹⁴. DMRIE-C (Life Technologies, Rockville, MD) was used to increase oligo uptake of cells. Transfection protocols were performed according to manufacturer's instructions.

Cell viability

To examine the effect of common chemotherapeutic drugs on FGFR3-expressing cells, therapeutic agents were added at increasing concentrations to the culture media containing B9-MINV (vector control), B9-WT (wild-type FGFR3) or B9-TD (activated mutant FGFR3) cells. Cells were analyzed 72 hours post-incubation using an Annexin V assay kit (Pharmingen, San Diego, CA) and the results were obtained by flow cytometry. MTT assays were also employed as per manufacturer's instructions (Promega, Madison, WI).

Western blots

Lysates were created and analyzed using western blotting techniques according to previously published procedures¹⁵. Membranes were probed with antibodies to Bcl-x_L and FGFR3 (C15) (Santa Cruz, Santa Cruz, CA). Goat anti-rabbit IgG horseradish peroxidase (Pharmingen, San Diego, CA) was used as the secondary antibody, and blots were developed by enhanced chemiluminescence according to manufacturer's instructions.

RESULTS and DISCUSSION

To determine if FGFR3 overexpression could confer chemoresistance we used three cell lines previously generated in our lab¹¹. All cell lines are derived from the murine IL-6-dependent myeloma cell line B9. These cells were infected with an empty retrovirus vector control (B9MINV) or the same retrovirus vector containing either the wild-type human FGFR3 gene (B9-WT) or a constitutively active form of FGFR3 found in Thanotophoric dysplasia (B9-TD). We treated all three cell lines with chemotherapeutic drugs commonly employed for the treatment of MM. Following exposure to Melphalan (Mel), or Doxorubicin (Dox) all cell lines responded in a similar manner, with significant cell death at relatively low levels of drug (Figure 1A).

In contrast, when treated with Dexamethasone (Dex), all of the cell lines were resistant (data not shown). Since B9 cells are IL-6-dependent, we believed that the observed Dex resistance likely reflected protective factors that were up-regulated by IL-6 and that such effects obscured any role for FGFR3. We, therefore, starved B9 cells of IL-6 for 24 hours in the presence of Dex, and then rescued surviving cells with IL-6. Following Dex treatment, cells ectopically expressing FGFR3 remained resistant to Dex, while parental and vector-only control cells were now sensitive to Dex-induced cell death (Figure 1A). Staining with acridine orange and ethidium bromide visually demonstrates an increase in cell viability in Dex-treated FGFR3-expressing cells (Figure 1B).

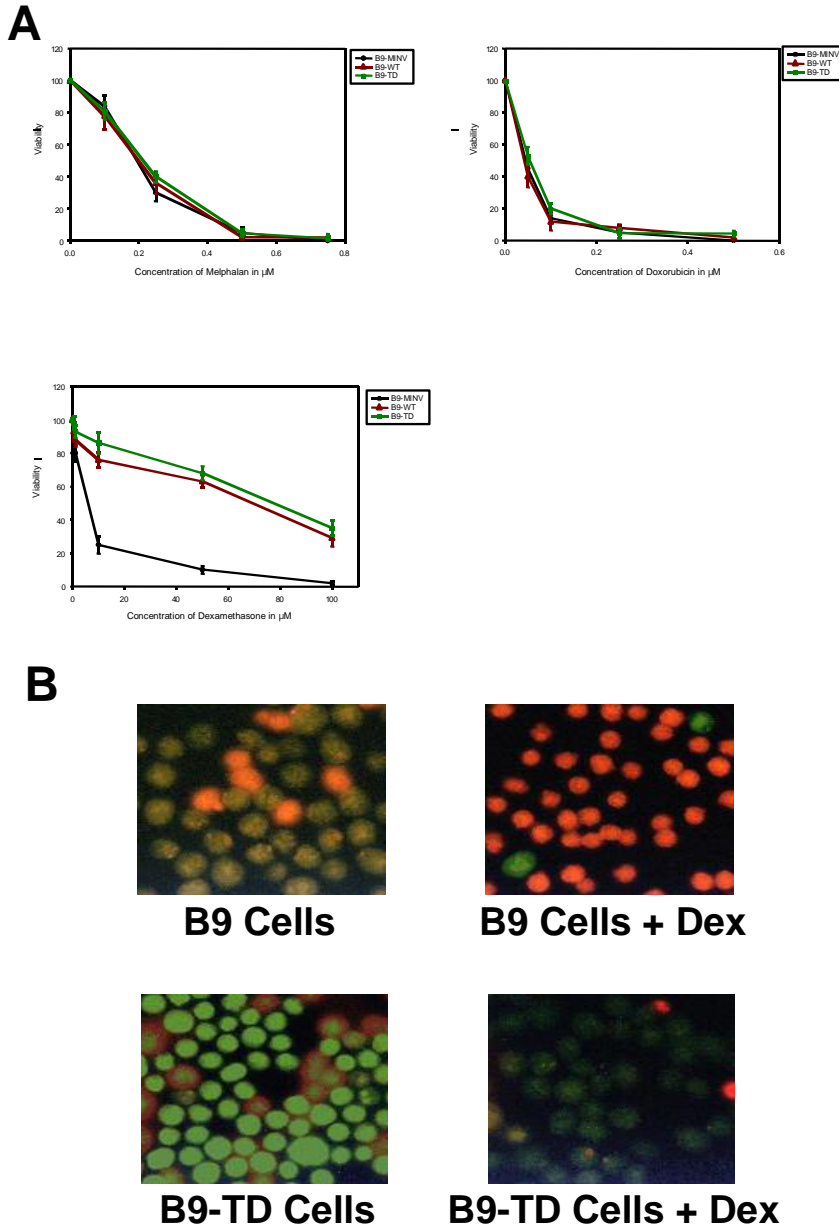


Figure 1. Treatment of FGFR3-Transduced Cells With Melphalan, Doxorubicin and Dexamethasone. (A) B9MINV, B9-WT and B9-TD cells were induced with various concentrations of drug and analyzed using an MTT assay kit at 72 hours post-induction. Elevated expression of FGFR3 confers resistance to Dexamethasone. (B) Ethidium Bromide/Acridine Orange Stained (green colour represents viable cells) B9MINV and B9-TD cells either untreated or treated with 10 μ M

Dex and rescued with IL-6. Only B9MINV cells are sensitive to Dexamethasone treatment.

Since Dex resistance appears to be correlated with FGFR3 expression levels, we next examined the sensitivity of B9 clones expressing high or low levels of wild-type FGFR3 with corresponding variable Bcl-x_L levels (Figure 2A). When treated with Dex, IL-6-starved high FGFR3-expressing B9 clones remained drug resistant, while the low FGFR3-expressing cells behaved in a pattern more indicative of the B9MINV controls (Figure 2B). Therefore, it appears that Dex resistance in B9 cells is at least, in part, related to FGFR3 expression levels.

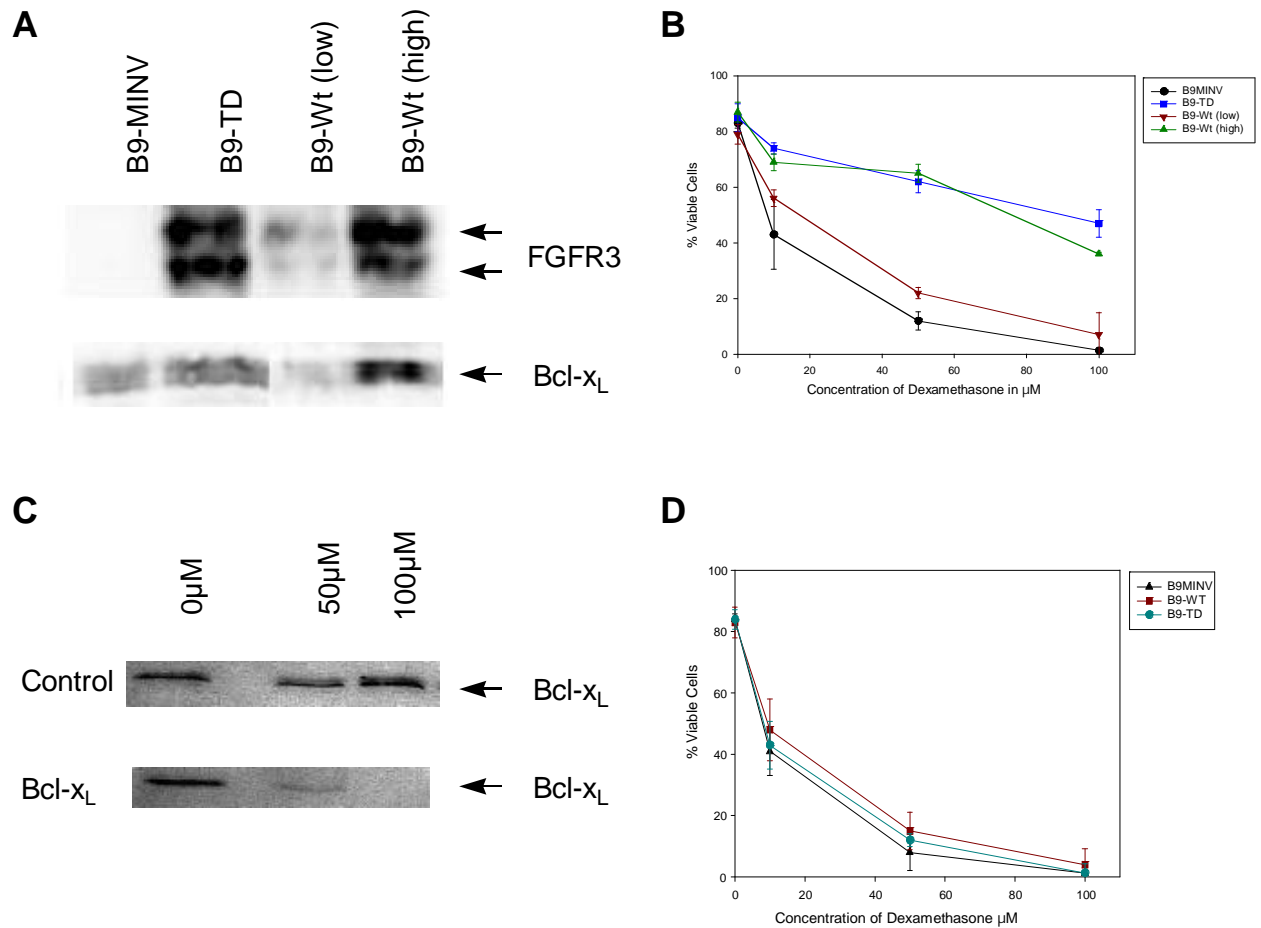


Figure 2. FGFR3-Overexpressing Cells are Resistant to Dexamethasone Due to Bcl-x_L Upregulation. (A) Western Blot analysis of high and low expressing FGFR3 clones. (B) Treatment of high and low FGFR3-Transduced Cells With Dexamethasone rescued with IL-6 and analyzed using annexin V assay kit at 72 hours post-treatment. This demonstrates a correlation between FGFR3 and Bcl-x_L levels and Dexamethasone resistance. (C) Western Blot analysis of B9-TD following treatment with control and antisense oligonucleotides demonstrates successful downregulation of Bcl-x_L. (D) B9MINV, B9-WT and B9-TD cells treated with various concentrations of Dexamethasone following Bcl-x_L antisense treatment and analyzed using annexin V

assay kit at 72 hours post-treatment. The protective effect of FGFR3 expression is abrogated.

In an earlier paper, we demonstrated that cells which overexpress either wild-type or the constitutively active FGFR3 gene maintain high levels of Bcl-x_L during IL-6 withdrawal¹¹. Thus, we hypothesized that Dex resistance reflects the maintenance of Bcl-x_L levels post-IL-6 withdrawal. To examine this hypothesis B9-FGFR3 cells were subsequently pre-treated with a Bcl-x_L antisense oligonucleotide 24 hours prior to addition of Dex. Under these conditions we demonstrated that the previously resistant FGFR3 cells became sensitive to Dex (Figure 2C, 2D). This data demonstrates that FGFR3-overexpressing cells become chemoresistant to Dex, at least in part, through the up-regulation of Bcl-x_L.

IL-6-induced Dex resistance has also recently been reported in ANBL6 (an IL-6-dependent human myeloma cell line), in that IL-6 is able to protect ANBL6 cells from Dex- and Mel- but not Dox-induced apoptosis¹⁶. Changes in ras or p53 expression can also alter ANBL6's response to IL-6¹⁶. In this regard, FGFR3 has also been reported to be a target for activating mutations that enable FGFR3 to play a ras-like role in tumor progression¹⁷. Mutations of ras and FGFR3 in human myeloma also appear mutually exclusive, and both ras and FGFR3 signaling lead to MAPK activation¹⁷. Thus, both FGFR3 and ras appear to alter IL-6 responsiveness and potentially confer Dex resistance.

We next investigated several human IL-6-independent myeloma cell lines with known FGFR3 translocations. Four of seven t(4;14) cell lines tested were resistant to Dex (OPM1, OPM2, KHM11 and H929), even at very high levels of drug (data not shown). The three cell lines which were Dex sensitive were re-tested following

stimulation with FGF ligand. FGF treatment abrogated Dex sensitivity in two (LP1 and UTMC2) of the three FGFR3-positive cell lines assayed in this manner (data not shown), but not in the cell line KMS11. Thus, FGFR3 stimulation reverses Dex sensitivity in at least some human myeloma cell lines. Studies in primary myeloma cells will be required to confirm this observation.

These results support the need to define the prognostic significance of IgH translocations in myeloma and suggest that tailored therapeutic approaches may be required. The correlation between FGFR3 translocations and clinical resistance to Dexamethasone has yet to be determined.

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