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13. ABSTRACT (Maximum 200 Words)

In the past year, I focused my research on testing the hypothesis is that NF-kB activation in breast cancer cells plays a critical role in cancer cells' resistance to anti-cancer drugs and to TNFα treatment, and MEKK3 is an essential component of this process. I constructed expression vectors that express dominant negative forms of MEKK3 that will be used to block the MEKK3-down stream cascades. I tested their expression and activities in various cells and found that the transfection efficiency in breast cancer cells is very low as compared to other cell lines such as COS-1 and 293T. In order to utilize these constructs in breast cancer cell, it is necessary to make retroviral vectors. This work is currently in progress. The second part of my research involves assaying NF-κB activation since it is the key target of MEKK3. I tested the NF-κB reporter activation, IkBa degradation, and NF-κB DNA binding activity. Finally, I have been working on the siRNA technique so that we can use MEKK3 siRNA to inhibit the MEKK3 expression in breast cancer cells, and then test whether this will render the cancer cells sensitive to TNFa and hopefully to other anti-cancer drugs as well.

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Role of MEKK3 signaling pathway in the resistance of breast cancer cells to TNFα-mediated apoptosis

Introduction

Breast cancer is the most commonly diagnosed malignancy and one of leading causes of death in American women. To date, the chemotherapy and radiotherapy are still common treatments for breast cancer. However, the efficiency of the treatment usually has been limited because breast cancer develops resistance to chemotherapeutic drugs, ionizing radiation, and tumor necrosis factor (TNF). TNFα is one of the most pleiotropic cytokine acting as a cytotoxic agent against a variety of tumor cell lines and also play a role in tumor regression mediated by cytotoxic T cells. TNFα is released by cytotoxic T cells and significantly contributes to the local immune response to the tumor. Tumor cells including breast cancer cells were naturally or acquire resistance to TNF-mediated apoptosis yet the mechanism is still not fully understood. We recently created MEKK3 knockout mice to investigate its in vivo function. We found that MEKK3 play a crucial role in TNF induced NF-κB activation and apoptosis (Yang et al. 2001). NF-κB activity induced by TNF was severely impaired in MEKK3-disrupted MEF cells but UV-induced NF-κB response was normal. Interesting, $MEKK3^{-/-}$ MEF cells are sensitive to TNF-induced apoptosis. This suggests that MEKK3 may be involved in breast cancer cells' resistance to TNF-mediated apoptosis.

Body

To test this hypothesis, we have been working on the conditions to altering the MEKK3 activities in normal and in breast cancer cells and then determine how TNF-responses will be affected. Two different strategies are being used, one is to use dominant interfering mutants to block the MEKK3 in breast cancer cells, and the second is to develop siRNA technique, a highly specific and efficient way to knock-out endogenous gene.

Key research accomplishment

Key accomplishments are:1) Generated three different MEKK3 mutants with mutations at the sub-kinase domain VII-III. Expressed these mutants in cell lines and detected their expression by western blot (fig. 1); 2) Constructed the retroviral expression vectors for these mutants (Naviaux et al. 1996), wild type MEKK3 and another dominant mutant MEKK3KM (fig 2); 3) determined NF-κB activation by TNF and other stimuli by IκBα western blot, by NF-κB reporter assay, and by gel-mobility shift assay (fig 3); 4) Constructed siRNA vector and tested its efficiency in cell lines (fig 4). One of the constructs will be further subcloned to lentiviral vector (Qin et al. 2003). 5). We prepared peptide antibodies against MEKK3 and in the process of optimize the condition for immunoprecipitation and western blotting with these antibodies.

Reportable outcomes

Although breast cancer cells are capable of being transfected, the efficiency is too low. Therefore we are testing the retroviral infection and then determine whether the MEKK3 pathway could be blocked. In addition to the two peptide-antibodies that were prepared previously, we generated additional anti-MEKK3 peptide antibody because the existing ones have extremely low titer and did not work for western blot. The new antibody was raised by a different company and preliminary data showed a promising result (fig 5).

We also tested one of siRNA construct and found the selected sequence appeared working to block transfected MEKK3.

Conclusions

I have constructed several expression vectors that express dominant negative mutant forms of MEKK3 that will be used to block the MEKK3-down stream cascades. I have tested their expression and activities in various cells and found that their transfection efficiency in breast cancer cell lines is very low as compared to other cell lines such as COS-1 and 293T. In order to utilize these constructs in breast cancer cell, it is necessary to make retroviral vectors. This work is currently in progress. The second part of my research involves assaying NF-κB activation. I have performed several different assays to measure the NF-κB activation since this is the key target of MEKK3. I have tested the NF-κB reporter activation, TNF-induced IκBα degradation, and NF-κB DNA binding activity. Finally, I have been working on the development of siRNA technique so that we can inhibit the MEKK3 expression in breast cancer cells, and then test whether this will render the cancer cells sensitive to TNFα and hopefully to other anti-cancer drugs as well.

Reference

Naviaux, R.K., E. Costanzi, M. Haas, and I.M. Verma. 1996. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J Virol* **70**: 5701-5.

Qin, X.F., D.S. An, I.S. Chen, and D. Baltimore. 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci U S A* **100**: 183-8.

Yang, J., Y. Lin, Z. Guo, J. Cheng, J. Huang, L. Deng, W. Liao, Z. Chen, Z. Liu, and B. Su. 2001. The essential role of MEKK3 in TNF-induced NF-kappaB activation. *Nat Immunol* **2**: 620-4.

Appendices fig 1-fig 5

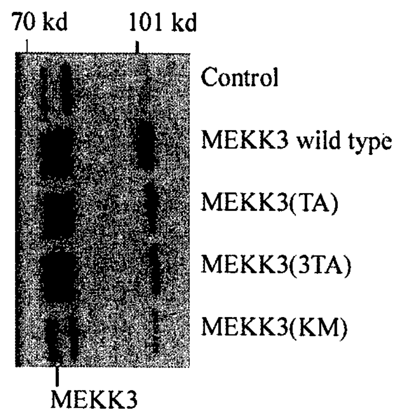

Figure 1 Expression HA-tagged wild type and mutant MEKK3. One microgram of empty vector of SRaHA-MEKK3 expression vectors were transfected into 293T cells and cell lysates prepared 36 h later for western blot analysis with anti-HA antibody as indicated.

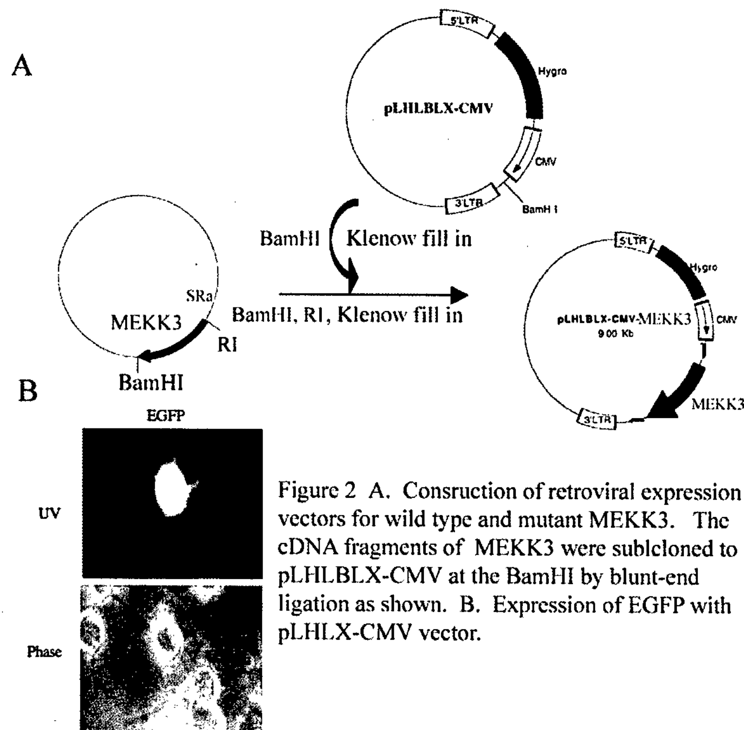

Figure 2 A. Consruction of retroviral expression vectors for wild type and mutant MEKK3. The cDNA fragments of MEKK3 were sublcloned to pLHLBLX-CMV at the BamHI by blunt-end ligation as shown. B. Expression of EGFP with pLHLX-CMV vector.

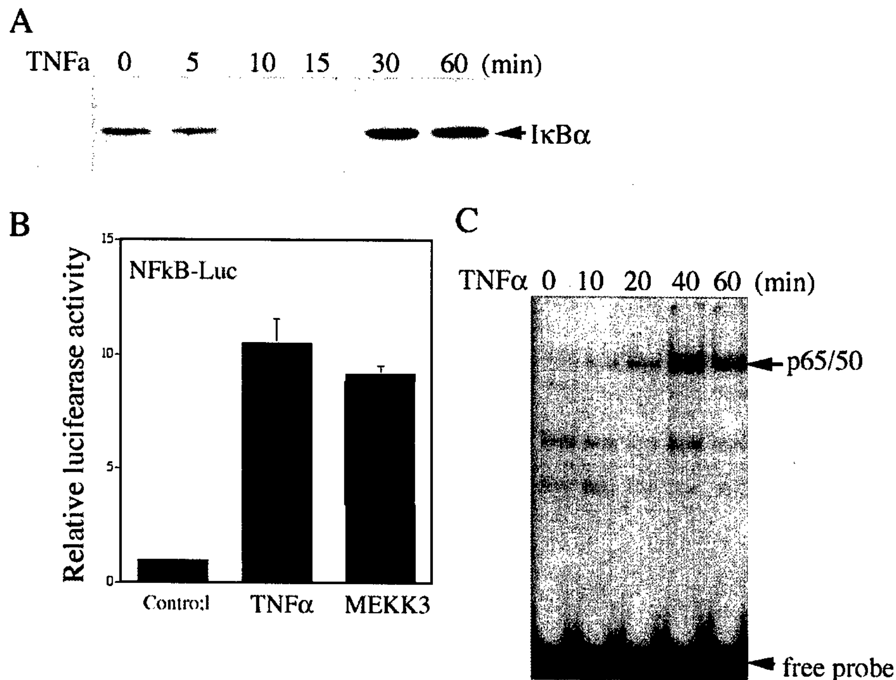

Figure 3 A. IκBα western blotting. Fibroblast cells either untreated or treated with TNFα for different time were lysed and analyzed by WB with anti-IκBα antibody. B. NF-kB reporter assay. One microgram of NF-κB-Luc reporter plasmid was transfected with either empty vector or with expression vector for MEKK3. Transfected cells were either unstimulated or stimulated with TNF for 12 h before being assayed for the

luciferase activity. C. NF-kB DNA binding assay. Two microgram of nuclear extract from control of TNFα satimulated cells were incubated with ^{32}P-labeled NF-kB oligo then analyzed by electrophoretic mobility shift assay (EMSA).

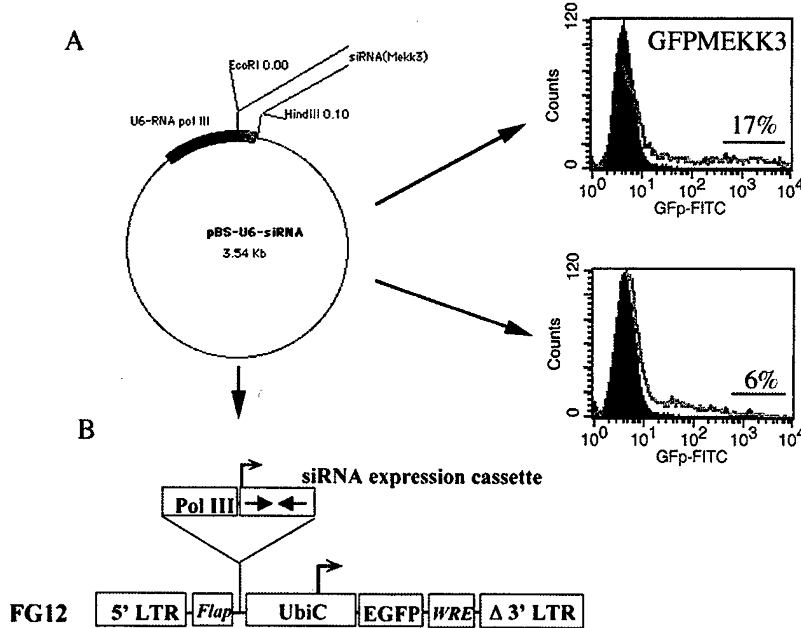

Figure 4 A. A control and a MEKK3 siRNA expression vector were co-transfected with MEKK3-GFP expression vector into BOSC cells and the GFP-MEKK3 fusion protein were analyzed 36 h later by flow cytometry. Decreasing of the fusion protein was indicated. B. Construction of lentiviral expression vector for the MEKK3 siRNA. The tested siRNA cassette will be subcloned into the lentiviral vector (Qin et al. 2003).

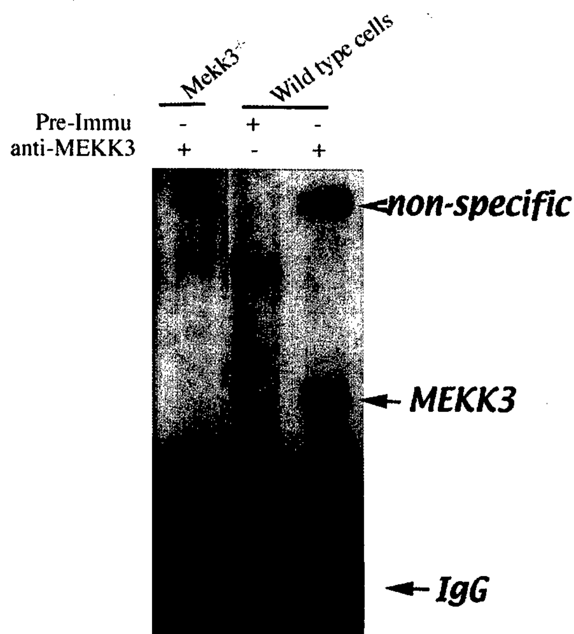

Figure 5 Immunoprecipitation-western blotting (IP-western) of endogenous MEKK3 with MEKK3-peptide specific antibody. Five million of 293T cells were lysed in 600 ul lysis

buffer and subjected immunoprecipitation with 1 ug of purified anti-MEKK3 peptide antibody. The immunocomplex was separated by a SDS-PAGE and subjected to western blotting with the same antibody at a concentration of 1 ug/ml. A pre-immune serum was used as a control for immunoprecipitation. Endogenous MEKK3 was indicated. Same amount of lysate from a MEKK3$^{-/-}$ fibroblast cells were used as a negative control.