Interaction between Nucleophosmin and the BCL-2 Family
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ABSTRACT
Nucleophosmin (NPM) is a nucleolar protein that translocates from nucleus to cytoplasm during cellular stress (Yun et al., 2003). NPM regulates cell homeostasis and maintains normal cell functions. Mutation of NPM is involved in many forms of Acute Myeloid Leukemia (AML). NPM may affect the apoptotic pathway via interaction with other apoptosis signaling proteins such as BCL-2 and BAX. In this study, a combination of protein pull-down techniques and Western blotting were used to study the interaction of NPM with BAX and BCL-2. Interaction of BCL-2 and rGST-NPM could not be verified because BCL-2 was detected in all samples with rGST beads. This research will aid in understanding the interactions between pro-apoptotic and anti-apoptotic proteins in the cytoplasm and further explain the mechanism of AML.

BACKGROUND
AML is a rapidly progressing disease characterized by accumulation of immature and abnormal myeloblasts in the bone marrow and by uncontrolled proliferation of these cells to other organs and tissues. Cytogenetic abnormalities are the frequent prognosis of AML (Byrd et al, 2002). Specifically, cytoplasmic localization or mutation of nucleophosmin (NPM) is involved in the pathogenesis in 50-60% of AML cases (Falini et al., 2008). NPM is a 37 kDa nucleolar protein that translocates from the nucleus to cytoplasm during cellular stress (manuscript in preparation). However, the cytoplasmic mutant form of NPM is largely localized to the cytoplasm. NPM is involved in many nuclear functions including ribosome assembly and transport (Olson et al., 1986), centrosome duplication (Okuda et al., 2000), molecular chaperone activity (Szebeni and Olson, 1999), regulation of p53 stability, and regulation of chromosomal stability and DNA repair (Li et al., 2006). Overexpression and mutation of NPM is frequently associated with human tumorigenesis (Grisendi et al., 2006). Through its interaction with members of the apoptotic signaling cascade, namely caspase-6 and -8, NPM may protect against cell death through retardation of death signal flow (manuscript in preparation). NPM is also shown to confer resistance to apoptosis (Li et al., 2000). Apoptosis is the programmed destruction of cells. Regulation of apoptosis affects growth and development of cells. The mitochondrial pathway of apoptosis is characterized by the release of molecules that normally reside between the outer and inner mitochondrial membranes into the cytosol by mitochondrial membrane permeabilization (MOMP), which is regulated by the members of the BCL-2 family (Chipuk, 2008). With four BCL-2 homology domains (BH1-4), the BCL-2 family can be categorized into anti-apoptotic proteins, which possess domains BH1-4, and proapoptotic proteins, which contain domains BH1-3 or only BH3.
BCL-2 is a 26 kDa anti-apoptotic protein. It plays an important role in survival of many cancer cells including AML by inhibiting the activation of pro-apoptotic proteins like the Bcl-2-associated X (BAX) (Konopleva, 2000). BAX is a 21 kDa cytoplasmic pro-apoptotic protein in the BCL-2 family. Upon activation, BAX inserts itself into the outer mitochondria membrane and increases MOMP (Chipuk et al., 2004). Apoptogenic factors such as cytochrome c and AIF from the mitochondria are then released from the mitochondria into the cytoplasm and apoptosis is committed (Leber et al, 2007). NPM has been identified as an important player in Bax binding protein and this interaction was speculated to be a crucial event in the activation and translocation of Bax in mitochondrial dysfunction and apoptotic cell death (Thompson et al, 2008).

In AML, mutated cytoplasmic NPM may perturb mitochondria death signaling through excessive interaction with BCL-2 or BAX. The purpose of this study was to examine the impact of excessive NPM level on physical interactions between the BCL-2 and Bax using a combination of protein pull-down techniques, and co-immunoprecipitation. The hypothesis is that NPM may interact with members of BCL-2 family and interferes with BAX inhibition to affect from MOMP and thus apoptosis.

Methods and Materials

Cell culture The Human Embryonic Kidney (HEK 293T) cell line was a gift from A/P Low Boon Chuan, NUS. HEK 293T cells were cultured in RPMI 1640 (Sigma) with 10% fetal bovine serum and 1% penicillin/streptomycin, grown in a humidified atmosphere of 5% CO2 at 37°C, and passaged at 90% confluence. Cells were collected by centrifugation, washed with ice-cold phosphate buffered saline (PBS), and lysed and collected with the mitochondrial/cytosolic fractionation kit following manufacturer’s instructions.

Co-immunoprecipitation of BAX 40μg of affinity purified antibodies against BAX (Cell Signaling Technolgies) was conjugated to 100μl AmidoLink Plus Coupling Resin from the Pierce Biotechnology Seize Primary Immunoprecipitation Kit according to manufacturer’s instructions. 400μg of cytosolic fractions were incubated with the conjugated resin and rotated overnight at 4°C. The resin was washed following manufacturer’s protocols and samples were eluted and concentrated with centrifugal concentrators (Sartorius) before SDS PAGE and Western Blot analysis.

Expression and purification of recombinant protein in E. coli Polyhistidine (His) tag-expressing plasmid pET32 containing either the gene for BCL-2 (His-BCL-2) or nucleophosmin (His-NPM) and glutathione S transferase GST tag-expressing plasmid pGEX4T containing the gene for BCL-2 (GST-BCL-2) were obtained from previously prepared (no need to mention who) and were transformed into E. coli strain BL21 (DE3) and strain DH5α, respectively. After growth in LB media the bacteria were induced with 1mM isopropyl beta-D-thiogalactoside (Biorad) for 3 h shaking at 25°C to express the recombinant protein. Bacteria were collected by centrifugation and resuspended and sonicated (Vibracell, 9s on/off pulse, 40% amplitude) in 10ml lysis buffer (1x PBS, 1% DL-dithiothreitol (DTT) (Promega), 1% protease inhibitor (PI) cocktail mixture (Roche Molecular Biochemicals), 1% Triton X-100). The supernatant was separated by centrifugation and glutathione-Sepharose (GE Healthcare) and Ni NTA (Qiagen) affinity purification beads were added to capture their respective binding partners. Upon rotation overnight at 4°C, centrifugation was performed and supernatant was discarded. Ni NTA for His tag and 1x PBS, 1% Triton-X100 was added for affinity purification. Supernatant
was removed by centrifugation at 55000 rpm. To elute purified proteins, elution buffer was added and the solution was incubated at room temperature for 5 min. Eluted proteins were concentrated with centrifugal concentrators (Vivaspin) and quantified using Bradford assay (Biorad). Purified proteins were separated by SDS-PAGE and visualized with Coomassie Blue.

**Cytosol fractionation**  
15 x 10^7 HEK 293T cells (one plate contains about 10^7) were harvested by centrifugation and washed with 10 ml ice-cold PBS. Cytosolic fraction was isolated using the Biovision Mitochondria/Cytosol fractionation kit following manufacturer’s protocol. Cytosol protein concentration was quantified by Bradford assay. The isolated cytosol was kept on ice and used within 4 hrs after cell lysis.

**GST- and His tag – pulldown of BAX**  
Equal amounts of recombinant GST-BAX (Abnova), GSTBCL-2, and His-BCL-2 and GST-NPM bound to their respective affinity beads were incubated with cytosolic fraction of HEK 293T cells at 4°C for 1 h. The beads were washed with PBS containing 0.1% Triton X-100 three times and collected by centrifugation before SDS-PAGE and Western Blotting analysis for bound proteins.

Electrophoresis and Western blotting analysis  
Samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 3% w/v skimmed milk for 15 min in Tris buffered saline with 0.1% v/v Tween-20 (TBST), followed by incubation with 1:1000 anti-BCL-2 (Cell Signaling Technologies), anti-BAX (Cell Signaling Technologies), and anti-NPM (Zymed) overnight. Membranes were then incubated in HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Santa Cruz, 1:1000) for one h and protein bands were visualized with Pierce Biotechnology SuperSignal West Pico chemiluminescent substrate, following manufacturer’s recommendations.

**Results and Discussion**

To study whether NPM affects BAX and BCL-2 binding, it is essential to first establish that BAX does indeed interact with BCL-2. Anti-BAX from Pharmigen, Cell Signaling, Chemicon, and Santa Cruz were tested. No BAX and BCL-2 interaction was detected with co-immunoprecipitation. Antibodies from Chemicon and Santa Cruz resulted in clearer blots whereas those from Pharmigen and Cell Signaling resulted in nonspecific signals. Pulldown assays with polyhistidine and glutathione S transferase tagged recombinant proteins were employed as alternative methods to testify interaction of BAX-BCL-2. Recombinant His-BCL-2 (rHis-BCL-2), His-NPM (rHis-NPM), and GST-BCL-2 (rGST-BCL-2) was produced in E. coli. The expression and purity of protein production was detected by electrophoresis and Western blotting analysis. The rHIS-BCL-2 production contained impurity with several unidentifiable bands that indicated non-specific binding or partially degraded proteins. rHis-BCL-2, rHis-NPM, and rGST-BCL-2 were incubated with cytosol fraction to pull down endogenous cytosolic BAX or recombinant GST–BAX (rGST-BAX). In the result of endogenous BAX pulldown, BCL-2 was probed and detected in all samples that contained rGST. This may be an indication that either endogenous BCL-2 binds to rGST tags or that the probe for BCL-2 resulted in nonspecific binding. Interaction of BCL-2 and NPM could not be confirmed because of the presence of BCL-2 in the rGST. Nevertheless, this experiment proved that BCL-2 was indeed present in the beads and that anti-BCL-2 successfully probed for BCL-2. The aim of this research was to analyze the role of NPM with comparison of the co-IPs with
and without NPM. Association of NPM with BAX-BCL-2 interaction was to be indicated by Western Blot analysis. This experiment was unable to perform since anti-BAX failed to depict BAX-BCL-2 interaction in and the pulldown assay for BAX was unsuccessful. As a result, the role of NPM in interfering BAX-BCL-2 interactions has yet to be determined.

References