Overexpression of Apg-2 increases cell proliferation and protects from oxidative damage in BaF3-BCR/ABL cells

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Abstract. Apg-2, a mammalian heat-shock protein belonging to the heat-shock protein 110 (Hsp110) family, was previously found to be overexpressed in BaF3-BCR/ABL cells that were treated with hydrogen peroxide (H_2O_2) through our comparative proteomics study. The expression of Apg-2 in chronic myelogenous leukemia (CML) cells and its role have not been investigated, forming the basis for this study. BaF3-MIGR1 and BaF3-BCR/ABL cell lines stably overexpressing Apg-2 were established and exposed to 50 μM H_2O_2 for 10 min. Western blot analysis of Apg-2 expression confirmed that H_2O_2 treatment significantly up-regulated Apg-2 expression. Apg-2 overexpression elevated BaF3-BCR/ABL cell proportions in S and G_2/M phase, increased cell proliferation and colony formation *in vitro*. Moreover, BaF3-MIGR1 and BaF3-BCR/ABL cells were exposed to 50 μM H_2O_2 in the absence or presence of Apg-2 overexpression and induction of H_2AX phosphorylation, the reporters of DNA damage were assessed by Western blot and immunofluorescence. Results showed that exposure to H_2O_2 induced H_2AX phosphorylation in BaF3-MIGR1 cells, but no increase was observed in BaF3-BCR/ABL cells. Together, the data indicate that Apg-2 is overexpressed and overexpression of Apg-2 in BaF3-BCR/ABL cells increases cell proliferation and protects cells from oxidative damage, which may play an important role in CML carcinogenesis and progression.

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Abbreviations: CML, chronic myelogenous leukemia; H_2O_2, hydrogen peroxide; Hsp, heat shock protein

Key words: Apg-2, hydrogen peroxide, overexpression, cell proliferation, H_2AX

Introduction

BCR/ABL protein with deregulated tyrosine kinase activity is encoded by the BCR/ABL gene, which is a result of the t(9;22)(q34;q11) chromosomal translocation. This fusion oncoprotein plays a central role in the pathogenesis of chronic myeloid leukemia (CML). Recently, imatinib mesylate (IM), which is an inhibitor of the BCR-ABL tyrosine kinase, has shown promise in treating CML patients (1). However, early relapses and IM-resistance have emerged as significant clinical problems in some IM-treated CML patients (2). Thus, it is critical to identify other therapeutics to prevent relapses and acquisition of resistance.

Heat-shock proteins (HSPs), also called stress proteins, are highly conserved proteins whose expressions are induced by various stresses (3). Recent studies have shown that overexpression of Hsp27, Hsp70, Hsp60 or Hsp90 inhibited apoptosis and prevented caspase activation in many different cellular models upon a variety of cellular stresses, including accumulation of misfolded proteins, reactive oxygen species (ROS) or DNA damage (3-6). Wang *et al* (7) also reported that Hsp110, Hsp70 and Hsp25 formed a large complex and directly interacted with one another in cell proliferation, cell cycle and protection from environmental stress. In addition, it is well known that Hsp70, one of the ATP-dependent molecular chaperones (8), is overexpressed in a multitude of cancers and is believed to play a role in tumorigenesis (9) DNA repair (10-12) and imatinib resistance in CML (13).

Apg (ATP and peptide-binding protein in germ cells)-2, a member of Hsp110 subfamily belonging to the Hsp70 family (14), was recently cloned from a mouse testis cDNA library using Apg-1 cDNA as a probe (15). Interestingly, Apg-2 protein shows a chaperone-like activity similar to Hsp110 and is overexpressed in hepatocellular carcinomas (16) as well as in pancreatic cancer (17), indicating that Apg-2 may be critical to tumor progression. In our previous comparative proteomics study, we found that Apg-2 was overexpressed in BaF3-BCR/ABL cells that were treated with hydrogen peroxide (H_2O_2) comparing to BaF3-MIGR1 cells (unpublished data). However, its underlying mechanisms are still unclarified.

Based on these studies, we hypothesized that overexpression of Apg-2 in BaF3-BCR/ABL cells that were treated with

H_2O_2 might contribute to cell proliferation, cell cycle and protection from environmental stress. In the present study, BaF3-MIGR1 and BaF3-BCR/ABL cell lines overexpressing Apg-2 were established and treated with H_2O_2. We demonstrated for the first time that Apg-2 was overexpressed in BaF3-BCR/ABL cells when treated with H_2O_2, which not only increased cell proliferation and colony formation, but also effectively protected cells from H_2O_2-induced injury.

Materials and methods

Cell culture and treatment. Murine pro-B cells BaF3 carrying MIGR1 vector (named BaF3-MIGR1) and $p210^{BCR/ABL}$ (named BaF3-BCR/ABL) were established by our group. The cells were grown in RPMI-1640 (Gibco-BRL, Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 ng/ml murine interleukin 3 (IL-3) but BaF3-BCR/ABL cells without IL-3 in a 5% CO_2 humidified atmosphere at 37˚C. Cells (~$2x10^6$) were harvested and treated with 50 μM H_2O_2 at 4˚C for 10 min.

Plasmid preparations and transfections. pCMV-Apg-2 containing full-length of murine Apg-2 cDNA was kindly provided by Professor Tongchuan He (Chicago Medical Center, USA). Full-length Apg-2 cDNA (GenBank accession number NM_008300) was digested with *Bgl*II and *Sac*II (New England BioLabs, Ipswich, MA, USA) and cloned to eukaryotic expression plasmid pIERS2-EGFP (preserved by our laboratory). Restriction sites were introduced by the forward primer (5'-GAAGATCTATGTCGGTGGTGGG CATAGACCTGGG-3') and the reverse primer (5'-TCC CCGCGGTCAATCAATGTCCATCTCAGGAAGCTT-3'). Sequence fidelity was confirmed by DNA sequencing. Cells (~$5x10^6$) plus 20 μg of plasmid DNAs were mixed in a Bio-Rad gene Pulser Cuvette and incubated at 4˚C for 10 min. Following one pulse electroporation (270 V, 960 μF), cells were incubated at 4˚C for 10 min and resuspended in RPMI-1640 plus 15% fetal bovine serum with 1 ng/ml murine interleukin 3 (IL-3). The transfected cells were first plated on 24-well dishes and selected in the presence of 800 μg/ml G418 at 37˚C, 5% CO_2 for 10 days. Then, selected resistant clones were expanded in 10-cm culture dishes for additional analysis.

Western blot analysis. Cells were washed with ice-cold PBS three times and lysed with lysis buffer. Protein concentrations were measured by modified Bradford assay. Protein extracts (~50-80 μg) were separated by 8-12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After membranes were blocked, followed by an overnight incubation with anti-Apg-2 (Santa Cruz, CA) and phospho-specific (*Ser*-139) histone H_2AX (γ-H_2AX) rabbit polyclonal antibody at 4˚C and then with the HRP-conjugated secondary anti-rabbit antibody (St. Louis, MO) at room temperature for 60 min. The immunoblots were revealed by autoradiography using enhanced chemiluminescence (ECL) detection kit (Millipore, Bedford, MA) and the band intensity was measured by densitometry using the Quantity One software (Bio-Rad Laboratories, Inc. Hercules, CA). The protein levels were normalized with respect to ß-actin protein level.

Cell cycle analysis. Cells were harvested and washed with ice-cold PBS and fixed with 70% ethanol overnight at 4˚C. Propidium iodine (10 μg/ml) supplemented with RNase A (200 μg/ml) was added to the cells for 30 min (at 37˚C) in the dark prior to FACS analysis (BD Biosciences, San Jose, CA). Percentages of G_0/G_1, S and G_2/M phase cells were determined using Modfit LT software (Verity Software House, Topsham, ME).

Cell proliferation assay. Cells (~$2x10^3$) were cultured in 96-well microplates (Falcon Plastic, CA, USA) using 100 μl optimal medium. At each time point (days 1, 3, 5, 7), 10 μl of SunBio™ Am-Blue (Shanghai Sun-Bio, China) was added to each well, and incubation at 37˚C and 5% CO_2 was continued for 6 h before measurement in triplicate for each time point. The relative fluorescent units (RFU) of living cells were detected by fluorescence microplate reader (CytoFluor, AB, USA) at different time points. RFU are directly proportional to number of living cells.

Methylcellulose colony formation assay. Cells were cultured in the methylcellulose media consisting of RPMI-1640 containing 0.9% methylcellulose (Aqua Solutions, Deer Park, TX), 20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin at the appropriate concentration and were plated at a density of 2000/ml in 1 ml volume in humidified 24-well plates. After 10 days of culture, the number of cell clones with >50 cells was counted under microscope in each well (clone formation rate = number of clones in each dish/2000). Three reduplicate wells were used from each clone.

Immunofluorescence. Cells were washed with ice-cold PBS three times. Cytospin slides of $2x10^5$ each group cells were prepared according to standard procedures and fixed in 100% prechilled methanol at -20˚C for 30 min. Following three washes with PBS (pH 7.2), slides were permeabilized with 0.3% Triton X-100/PBS for 15 min at 37˚C, blocked in 10% normal goat serum for 3 h at room temperature and incubated with indicated antibodies at 4˚C overnight followed by three washes with PBS (pH 7.2) containing 0.5% Triton-X 100 (Sigma, St. Louis, MO). The cells were further incubated with goat anti-rabbit PE-IgG, washed, and DNA was counterstained with DAPI for 3 min. After three washes with PBS, the cells were treated with a fluorescent mounting medium (Dako), and visualized by confocal microscopy (LEICALasertech GmbH, Heidelberg, Germany). Fluorescence images were acquired by means of a PL-APO x100 objective. In order to confirm the cellular localization in the 3-D images, 4-sectioned images were overlapped into a single image at the same phase.

Statistical analysis. Data were expressed as means ± SE. All the data were analyzed using the SPSS statistical package 13.0. Mean values were compared by unpaired t-test. $P<0.05$ were considered statistically significant.

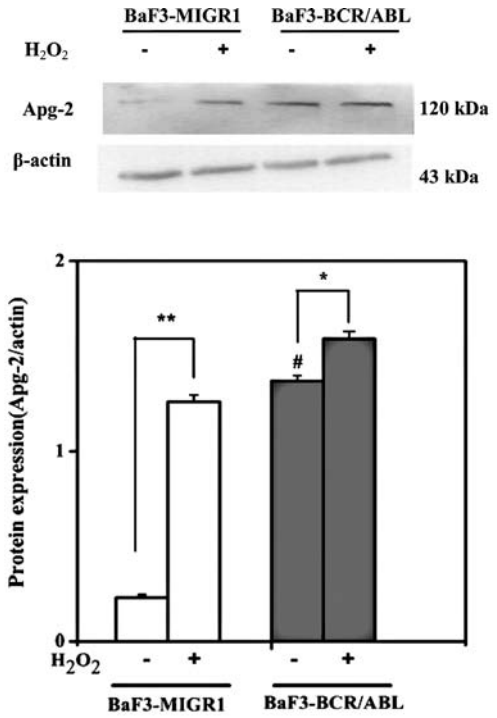

Figure 1. Western blot analysis of Apg-2 expression in cells exposed to H_2O_2. BaF3-MIGR1 and BaF3-BCR/ABL cells were exposed to 50 μM H_2O_2 at 4˚C for 10 min, then the cells were collected for Western blot analysis. Data are representative of three independent experiments. *p<0.05, **p<0.01 vs untreated cells; $^{\#}$p<0.05 BaF3-BCR/ABL cells vs BaF3-MIGR1 cells.

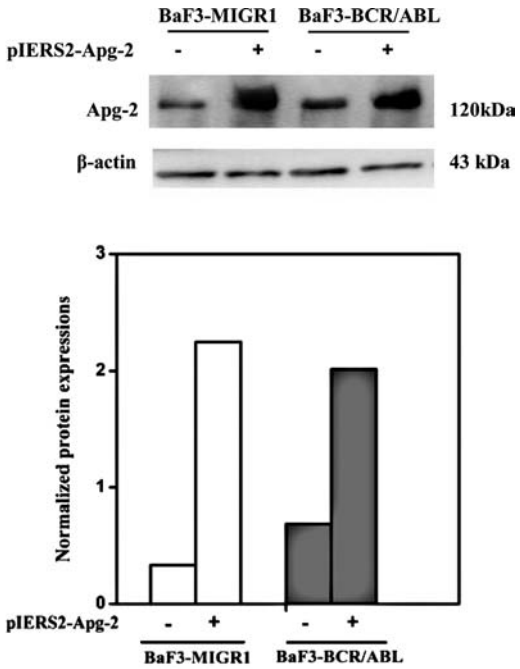

Figure 2. Western blot analysis of Apg-2 expression in cells transfected with pIERS2-Apg-2 gene. Apg-2 protein expression was analyzed by Western blot as described in the text. ß-actin is shown as internal standard for the amount of protein applied in the samples. The band intensity was measured by densitometry using the Quantity One software.

Results

H_2O_2 treatment accumulated Apg-2 expression in BaF3-BCR/ABL cells. H_2O_2 (50 μM) was applied to BaF3-MIGR1 and BaF3-BCR/ABL cells for 10 min, and Apg-2 expression was assessed by Western blot. As shown in Fig. 1, Apg-2 protein expression was up-regulated after exposure to H_2O_2, and BaF3-BCR/ABL cells displayed much higher level than BaF3-MIGR1 cells, which was lined with our previous comparative proteomics study (unpublished data). To investigate the potential role of Apg-2 in BCR/ABL-positive cells, both BaF3-BCR/ABL cell lines and the peer BaF3-MIGR1 cell lines for control stably overexpressing Apg-2 were constructed. Western blot analysis identified that the expression levels of the Apg-2 protein in the pIERS2-Apg-2-transfected cells were approximately three-fold higher than that in the controls (Fig. 2).

BaF3-BCR/ABL cells with Apg-2 overexpression had higher proportions of S and G_2/M phase. To monitor the effect of Apg-2 overexpression on the cell cycle, the DNA content of transfected cells was analyzed by FACS. The results showed that the proportion of BaF3-MIGR1-Apg-2 cells in G_0/G_1 phase was increased to 53.39±0.45% (P<0.05), while the proportion of BaF3-MIGR1-Apg-2 cells in S phase was reduced to 41.29±1.08% (P<0.05). On the other hand, BaF3-BCR/ABL-Apg-2 cells showed lower proportion in G_0/G_1 phase (20.87±0.38) and higher proportion in S phase and G_2/M phase (71.45±1.34% and 7.69±0.96%) compared with BaF3-BCR/ABL cells (Table I and Fig. 3). BaF3-BCR/ABL-

Table I. Cell cycle analysis by flow cytometry.

Group	G_0/G_1 (%)	S (%)	G_2/M (%)
BaF3-MIGR1	37.39±0.38	60.04±0.70	2.57±1.05
BaF3-MIGR1-Apg-2	53.39±0.45[a]	41.29±1.08[a]	5.32±1.53[a]
BaF3-BCR/ABL	28.27±0.25[b]	66.72±0.73[b]	5.01±0.99[b]
BaF3-BCR/ABL-Apg-2	20.87±0.38[a]	71.45±1.34[a]	7.69±0.96[a]

Cell cycle profiling by flow cytometric analysis of DNA content was performed on the overexpression cells and the control groups. Percentages of G_0/G_1, S and G_2/M phase cells were calculated with Modfit LT software. Results are the average of 3 experiments. Values are means (± SE). [a]P<0.05 vs untransfected cells; [b]P<0.05 vs BaF3-MIGR1 cells.

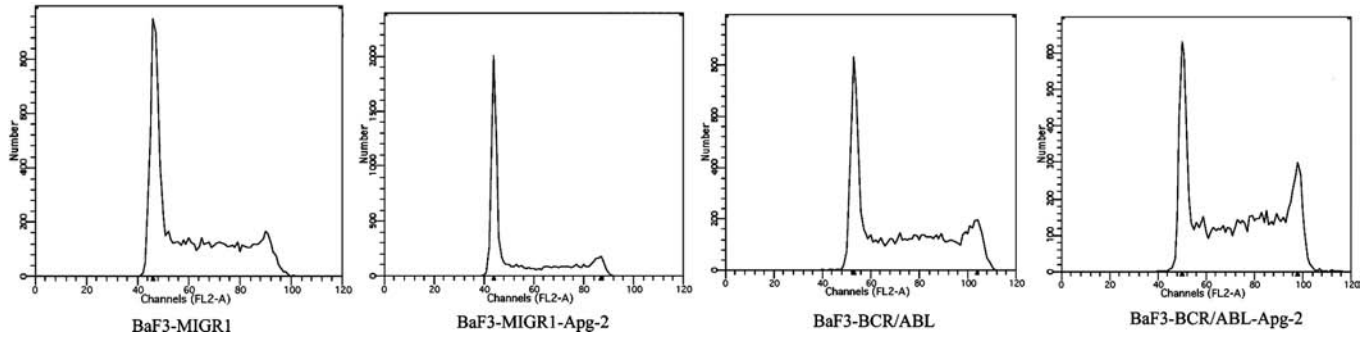

Figure 3. Cell cycle analyzed by FACS. Representative FACS images of BaF3-MIGR1, BaF3-MIGR1-Apg-2, BaF3-BCR/ABL and BaF3-BCR/ABL-Apg-2 cells were from three independent experiments.

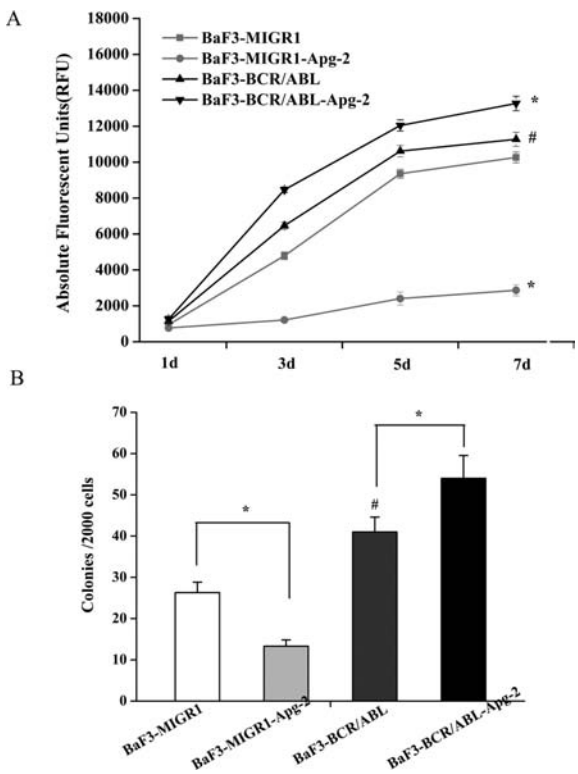

Figure 4. Cell proliferation and colony formation assays. (A) Effects of Apg-2 overexpression on cell proliferation. The details are described in the Materials and methods. Each point represents the mean ± SE for 3 wells. Data shown are representative of 3 experiments. (B) Colony formation was examined as depicted in Materials and methods. The number of colony was counted at light microscope. Results represent means ± SE of 3 separate experiments. *p<0.05 vs untransfected cells, #p<0.05 BaF3-BCR/ABL cells vs BaF3-MIGR1 cells.

untransfected cells also displayed increased proportion in S phase and G_2/M phase.

Apg-2 overexpression increased cell proliferation and colony formation in BaF3-BCR/ABL cells. The growth properties of cells with Apg-2 overexpression *in vitro* were evaluated. In comparison to BaF3-MIGR1 cells, BaF3-BCR/ABL cells and BaF3-MIGR1-Apg-2 cells displayed increased and decreased cell viability, respectively. However, BaF3-BCR/ABL-Apg-2 cells showed higher cell viability, as tested by Am-Blue assay (Fig. 4A). BaF3-MIGR1-Apg-2 cells formed markedly less colonies than did in BaF3-MIGR1 cells, but Apg-2 overexpression revealed a ~47% increase in colony formation in BaF3-BCR/ABL cells as compared with BaF3-BCR/ABL cells (Fig. 4B).

Apg-2 overexpression protected BaF3-BCR/ABL cells from H_2O_2-induced oxidative damage. Earlier studies have shown that γ-H_2AX, a phosphorylated form of histone H_2AX on *Ser*-139, is an early indicator of cellular DNA damage, particularly the damage that involves the formation of DNA double-strand breaks (DSBs) (18-20). Exposure to H_2O_2 resulted in induction of H_2AX phosphorylation (Fig. 5), which was reinforced by Apg-2 overexpression in BaF3-MIGR1 cells. But no response was elicited in BaF3-BCR/ABL cells either by Apg-2 overexpression or H_2O_2 treatment. The constitutive phosphorylation of H_2AX was also observed in untreated cells.

The induction of γ-H_2AX manifested in form of IF foci (Fig. 6). The microphotographs shown in this figure demonstrate formation of a large number of γ-H_2AX IF foci in

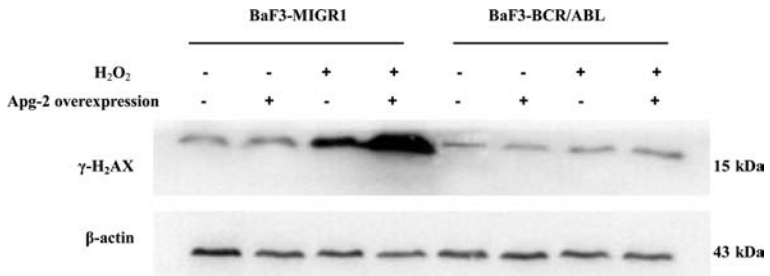

Figure 5. γ-H_2AX expression in cells exposed to H_2O_2. Cells untreated or treated with 50 μM H_2O_2 were analyzed by Western blot for measurement of γ-H_2AX expression.

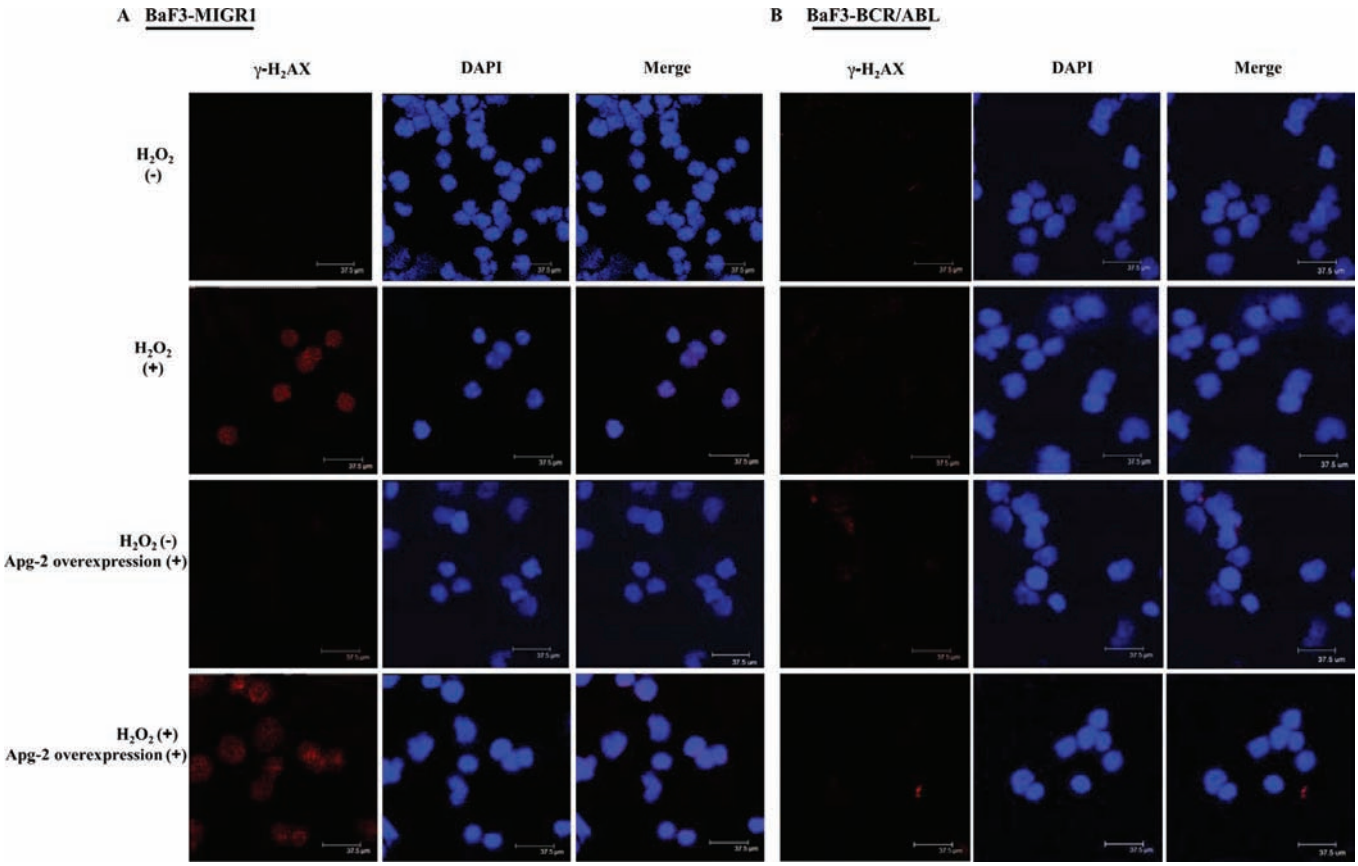

Figure 6. A and B, Confocal microscopic analysis of γ-H_2AX. Cells untreated or treated with 50 μM H_2O_2 were stained with anti-γ-H_2AX antibody and PE-labeled goat anti-rabbit IgG (red), followed by DAPI counterstaining (blue).

BaF3-MIGR1 cells that were exposed to H_2O_2 or Apg-2 overexpression (Fig. 6A). However, there was almost total suppression of γ-H_2AX IF foci formation in BaF3-BCR/ ABL cells that were exposed to H_2O_2 or Apg-2 overexpressed (Fig. 6B).

Discussion

This study demonstrated for the first time the following: a) H_2O_2 treatment leads to remarkable accumulation of Apg-2 expression in BaF3-MIGR1 and BaF3-BCR/ABL cells. b) Apg-2 is significantly up-regulated in BaF3-BCR/ABL cells as compared to that in BaF3-MIGR1 control cells. c) Apg-2 overexpression in BaF3-BCR/ABL cells elevates cell proportions in S and G_2/M phase and increases cell proliferation. d) Overexpression of Apg-2 in BaF3-BCR/ ABL cells suppresses H_2AX phosphorylation and γ-H_2AX IF foci formation. These results clearly indicate the important role of Apg-2 in BaF3-BCR/ABL cells.

Apg-2, a member of the Hsp110 family, is overexpressed in carcinomas and is therefore thought to play a role during proliferation (17,21-22). In the present study, we found that the expression of Apg-2 was increased when exposed to H_2O_2 and BaF3-BCR/ABL cells also showed a higher level of Apg-2 as compared with BaF3-MIGR1 cells. Moreover, overexpression of Apg-2 conferred a proliferative advantage to BaF3-BCR/ABL cells *in vitro*. In accord with our findings, a previous study has demonstrated that Apg-2 is overexpressed in hepatocellular carcinoma (16). Together, these findings suggested that Apg-2 is overexpressed when exposed to H_2O_2 in BaF3-BCR/ABL cells, which promotes cell proliferation.

The present data illuminated that exposure of BaF3-MIGR1 cells to H_2O_2 led to rapid induction of H_2AX phosphorylation. This observation is compatible with the known mechanism of DNA damage by oxidants that initially leads to formation of DNA single-strand lesions, predominantly consisting of 8-oxo-dG, followed by conversion of some of these lesions into DSBs during DNA replication (23-25). Consistent with the induction of DSBs by H_2O_2 was also observation of γ-H_2AX foci (Fig. 6). Therefore, the above evidence points out that in the cells treated with H_2O_2 the induction of γ-H_2AX reports oxidative damage.

Oxidative damage induced by H_2O_2 as reflected by induction of γ-H_2AX was distinctly reduced in BaF3-BCR/ ABL cells that were exposed to H_2O_2 in the presence of Apg-2 overexpression, which was similar to the level of γ-H_2AX expression on the H_2O_2-untreated cells. These results provide evidence for the protective effect of Apg-2 on BaF3-BCR/ ABL cells stressed by H_2O_2. As mentioned, the constitutive phosphorylation of H_2AX found in untreated cells reflects to a large extent the ongoing DNA damage induced by endogenous oxidants produced primarily in mitochondria during aerobic metabolism (26,27).

It is unclear why the cell proliferative advantage and protective effect on oxidative damage were only observed in BaF3-BCR/ABL cells with Apg-2 overexpression not in BaF3-MIGR1 cells. One of the mechanisms could be that Apg-2 had a chaperone-like activity (17), interacting with BCR/ABL. Previous study in radiated mice demonstrated that Apg-2 was involved in the induction of the radioadaptive response and played a role in the radiation-induced DNA damage (20). It is likely that Apg-2 interacting with BCR/ABL promotes cell proliferation and protects cells from oxidative damage, which may be associated with H_2O_2-induced DNA repair.

Collectively, this study suggests that Apg-2 is overexpressed in BaF3-BCR/ABL cells and the cells that were exposed to H_2O_2. Overexpression of Apg-2 promotes cell proliferation and protects against oxidative damage in BaF3-BCR/ABL cells. Studies for precise understandings of the underlying mechanisms are under way. It may be possible to clarify carcinogenesis and progression of CML and develop novel strategies for CML treatment.

Acknowledgments

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