Low-pH-induced apoptosis: role of endoplasmic reticulum stress-induced calcium permeability and mitochondria-dependent signaling

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Abstract The acidic microenvironment around tumor cells is a major determinant in cancer growth, metabolism, and metastasis. However, its role in cancer physiology is still not clearly understood. In the present investigation, an attempt has been made to explore the effect of acidic environment on physiology of cancer cells. Exposure of Raji cells to extracellular acidic environment was associated with enhanced cytosolic calcium level and endoplasmic reticulum stress response. X-box binding protein 1 (XBP1) splicing, CCAAT/enhancer-binding protein homologous protein (CHOP), and glucose-regulated protein 78 kDa (GRP78) upregulation suggested endoplasmic reticulum stress generation. On the other hand, real-time-based upregulation of *Bax* gene expression and flow cytometric analysis of cytochrome c release as well as enhanced active caspase-3 further confirmed mitochondrion-mediated events leading to induction of apoptosis. The expression of *TP53* and *p21* was upregulated. These observations collectively strongly suggest that both endoplasmic reticulum stress-mediated calcium release and *Bax* targeting might be altering mitochondrion membrane potential

which in turn could induce secondary apoptotic signals; subsequently, endoplasmic reticulum stress can also lead to nuclear localization of Nuclear factor- κ B (NF- κ B) which in turn favors p53 mediated apoptotic signals.

Keywords Acidic pH · Apoptosis · Calcium · Cytochrome c · Endoplasmic reticulum

Introduction

The body must maintain proper balance of acidity to alkalinity (pH) in the bloodstream in order to function properly. In cancer, exponentially growing cells receive poorly organized and only marginally functional vasculature. Due to increased glycolytic activity of malignant cells, insufficient vascular supply, and sluggish blood circulation, intratumor environment becomes acidic with pH less than 6.0 in certain tumors (astrocytomas and squamous cell carcinomas) as compared to a normal physiological pH of 7.3 (Schornack and Gillies 2003; Vaupel et al. 1989). In addition, low influx of metabolites and a low efflux of potentially toxic metabolic wastes further enhance the acidic conditions (Tannock and Rotin 1989). Acidic environment plays an important role in cell death during various pathophysiological states, including cancer (Williams et al. 1999). A shift to acidic pH within solid tumors can regulate proliferation, angiogenesis, immune suppression, invasion, and chemo resistance. Likewise, acidic environment or low pH can also influence oncogenesis, malignant transformation, and metastasis (Rofstad et al. 2006). The environmental acidity also greatly influences the response of cancer cells to various treatments. There are several contradictory reports in literature regarding the effect of acidic environment on

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physiology of cells; some studies suggest that low pH can promote tumor progression while others indicate that it can induce apoptosis in cancer cells (Hjelmeland et al. 2011; Park et al. 1999). It has been demonstrated that acidic stress can lead to the upregulation of Src activity, matrix metalloproteinases (MMPs), and VEGF expression, which in turn can promote tumor progression and metastasis (Chen et al. 2008; Kato et al. 1996, 2005; Xu et al. 2002; Fukumura et al. 2001). Studies have demonstrated that low pH has the ability to induce genes and pathways which collectively lead to progression of malignancy (Duggan et al. 2006). Bhat et al. (2012) have shown that acidic stress can promote oligomerization and membrane insertion of apoptosis repressor BclXL (Bhat et al. 2012). In contrast, Park et al. (1999) reported that acidic pH can promote the factors which can move the cancer cells physiology toward apoptosis; i.e., acidic pH can have therapeutic potential. Acidic pH-mediated induction of apoptosis was suggested to be a consequence of caspase activity (Park et al. 1999). Acidic environment could modulate growth as well as radiochemosensitivity of glioma cells (Reichert et al. 2002) and was demonstrated to have immunomodulatory activity by promoting the expression of IL-8 in cervical cancer cells (Xu and Fidler 2000). Hypoxia and acidity represent two factors that might be exploited therapeutically to destroy such cells. Recently, resveratrol and many drugs like lovastatin and cantharidin have been shown to be more effective in a low pH environment than at normal pH (Shamim et al. 2012; Fukumachi et al. 2010). It has been demonstrated that intracellular acidification by exposure to acidic environment or by alteration in intracellular pH control mechanisms leads to cellular damage and sensitizes cells to chemotherapy or hyperthermia (Haveman 1979; Chu and Dewey 1988). But, even after extensive research in this area, there is a dearth of information about molecular mechanism involved in various physiological responses under acidic environment and the therapeutic potential of acidifying tumor microenvironment.

Leukemia is cancer of blood cells, and it has been shown that acidic microenvironment is a key feature of exponentially growing cells in bone marrow (Mortensen et al. 1998). Especially, acute leukemia is a rapidly progressing disease that results in the accumulation of immature, abnormal cells in the marrow and blood. The metabolic activity of rapidly growing cancer cells could alter environment around the cancer cells in soft tissue and bone marrow. Therefore, in the present investigation, acute lymphoid leukemia Raji cells were selected as a suitable model to study the effect of acidic environment on leukemic cell physiology. It was observed that Raji cells exposed to extracellular acidic environment underwent apoptosis without any contribution of reactive oxygen species (ROS). Further, the growth of Raji cells in a medium of low pH resulted in an augmentation in intracellular calcium level and generation of endoplasmic reticulum (ER) stress that induced apoptosis by Bax targeting, cytochrome c release, and caspase

activation. Raji cells exposed to acidic stress also showed up-regulation of *TP53* and *p21* and nuclear localization of Nuclear factor- κ B (NF- κ B). In conclusion, the study highlights that alteration (lowering of pH) of tumor microenvironment can be used as a therapeutic option to suppress tumor growth.

Materials and methods

Cell culture and treatment

Exponentially growing Raji cells (human acute lymphoid leukemia cells) were procured from the National Centre for Cell Science (NCCS), Pune. The cells were cultured under a humidified 5 % carbon dioxide and 95 % air atmosphere at 37 °C. The cell density was maintained at fewer than 3×10^5 cells/ml in 25-cm² plastic tissue culture flasks with RPMI-1640 culture medium supplemented with 10 % (v/v) fetal calf serum. To determine the effect of low pH on Raji cells, cells were plated at a density of 0.5×10^6 cells/ml in culture media of pH 5.8 and 6.8 for 48 h and cells grown at physiological pH 7.3 were taken as control. pH values of 6.8 and 5.8 were selected based on the studies which suggested that tumor pH can vary from 6.8 to 5.8 (Song et al. 2006; Tannock and Rotin 1989).

Cellular proliferation assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. This method is based on the ability of viable cells to reduce MTT to form colored formazan product (Heckenkamp et al. 1999). Briefly, cells were cultured in 96-well plates at a density of 1×10^4 cells per well in media with different pH—pH 7.3, pH 6.8, pH 6.3, and pH 5.8. After incubation for 12, 24, and 48 h, MTT dissolved in phosphate-buffered saline (PBS) was added to each well at a final concentration of 5 mg/ml and then incubated at 37 °C and 5 % CO₂ for 2 h. The water-insoluble dark blue formazan was dissolved in DMSO. Optical density was measured by a microplate reader (Bio-Rad) at a wavelength of 570 nm.

DNA fragmentation assay

Along with control (pH 7.3), 0.5×10^6 cells/ml were cultured in acidic conditions (pH 6.8, pH 6.3 pH 5.8) for 48 h. DNA from treated cells was extracted according to Gong's modified method (Gong et al. 1994). Briefly, cells were washed twice in cold PBS (10 mM) and resuspended in hypotonic lysis buffer (5 mM Tris HCl, 20 mM EDTA, pH 7.4) containing 0.5 % Triton X-100 for 30 min at 4 °C. The lysate was centrifuged at 13,000g for 15 min at 4 °C. DNA was extracted from the supernatant with equal volume of phenol-chloroform-isoamylalcohol, precipitated by addition of 0.1 volume of

3 mM sodium acetate and two volumes of absolute ethanol. After treatment with RNase A (500 U/ml) at 37 °C for 3 h, the pattern of DNA fragmentation was analyzed on 1.5 % agarose gel.

RT-PCR and quantification of mRNA expression levels

Total RNA was isolated from treated and untreated cells using TRIzol reagent (Sigma, USA). One microgram RNA was used for complementary DNA (cDNA) preparation using Verso cDNA kit (Thermo Scientific, USA). DNA contamination in total RNA isolated was avoided using reverse transcription (RT) enhancer available with kit. Real-time PCR analysis was performed in Eppendorf realplex system using the SYBR Green PCR Master mix (Thermo Scientific, USA). Real-time PCR was carried out for *TP53* (Yang et al. 2013), *p21* (Fields et al. 2005), *Bax* (Sharma et al. 2012), and β -actin (Jha et al. 2010) using gene-specific primers. β -Actin served as an internal control. Specificity of PCR products was analyzed using melting curve analysis, and delta CT method was used to quantify alteration in expression.

Detection of XBP1 mRNA splicing

Total RNA was extracted from treated and untreated cells and subjected to cDNA preparation as described above. The cDNAs were PCR amplified using specific primers for *XBP1* (Zhu et al. 2012), CCAAT/enhancer-binding protein homologous protein (CHOP) (Wang et al. 2011), and glucose-regulated protein 78 kDa (*GRP78*) (Lin et al. 2012). The primers used were *XBP1F* (5'-GGTAAGGAAGTGGGTCCTT-3') and *XBP1R* (5'-GAGTTAAGACAGCGCTTGGG-3'). A 331-bp PCR product was amplified from the unspliced form of *XBP1* messenger RNA (mRNA), which contains the 26-bp intron, and a 305-bp PCR product was amplified from the spliced form of *XBP1* mRNA. The PCR products were separated on a 12.5 % polyacrylamide gel.

Cytochrome c release, active caspase-3, and p21 determination

Cells (0.5×10^6 cells/ml) were washed once in PBS and then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences, USA) for 20 min on ice. Cells were pelleted, washed with Perm Wash Buffer (BD Biosciences, USA), and stained with fluorochrome-conjugated antibody cytochrome c mAb (Santa Cruz Biotechnology, USA), rabbit anti-human active caspase-3 fluorescein isothiocyanate (FITC) mAb (BD Biosciences, USA), or p21 PE mAb (Santa Cruz Biotechnology, USA) at 4 °C

for 1 h. Cells were washed twice with Perm Wash Buffer and finally resuspended in Perm Wash Buffer for flow cytometry analysis. Flow cytometric analysis was performed on a BD FACS Canto II (BD Biosciences, USA) for a maximum cell count of 5000 and analyzed using BD FACS Diva software.

Cytosolic calcium and ROS determination

Cytosolic Ca^{2+} levels were determined using the fluorescent dye Fluo 3-AM (1 mM) (log mode in FITC setting). Treated and untreated cells were incubated with fluorescent dye for 15 min at 37 °C, washed with PBS containing 10 mM glucose, and analyzed immediately by flow cytometry. The intracellular accumulation of ROS was determined using 2',7' dichlorofluorescein diacetate (DCFH-DA) (Sigma, USA). After treatment, the cells were washed with PBS, stained with DCFH-DA for 20 min at 37 °C, and analyzed with flow cytometry for maximum cell count of 5000.

Western blotting

Cells were harvested and cell pellet was washed with cold phosphate-buffer saline. Cells were lysed in lysis buffer (20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 % NP-40, 1 mM ethylene glycol-bis(β -aminoethyl ether)-tetraacetic acid, 1 mM EDTA, 50 mM NaF, 1 mM β -glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM orthovanadate, one protease inhibitor cocktail, 1 mM PMSF), and protein content in the supernatant was determined by protein estimation kit (Bangalore Genie). Equal amount of cell lysate (200 μ g/well) from both treated (pH 6.8, pH 5.8) and untreated (pH 7.3) was resolved on 10 % SDS-PAGE followed by transfer onto a PVDF membrane. Membranes were probed with anti p53 (1:1000), Bax (1:1000), and NF- κ B (1:1000) antibodies (Sigma, USA). Horseradish peroxidase-conjugated secondary antibodies were used to detect immune-reactive bands using 3, 3'-diaminobenzidine as substrate.

Subcellular fractionation

After treatment, Raji cells were suspended in hypotonic buffer A (250 mM sucrose, 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 100 μ M PMSF) on ice and incubated for 30 min followed by pipetting for 30 min. Cell lysate was centrifuged at $8700 \times g$ for 10 min at 4 °C to remove unlysed cells and nuclei. The supernatant was centrifuged at $43,000 \times g$ for 5 min at 4 °C. The pellet was saved as mitochondrion fraction followed by four times washing of pellet with lysis buffer. With the Pasteur pipette, supernatant was recovered and centrifuged at $110,000 \times g$ for 1 h at 4 °C. The supernatant was saved as cytosolic fraction and the

Fig. 1 Acidic stress-induced apoptosis in Raji cells. **a** Percentage viability of Raji cells grown in media of pH 7.3, 6.8, 6.3, and 5.8 after 12, 24, and 48 h. **b** Detection of apoptosis by DNA laddering assay. Raji cells were grown in acidic environment (pH 6.8, pH 6.3, and pH 5.8) for 48 h, and the cells grown at pH 7.3 were taken as control

pellet as ER/microsome fraction. To further purified ER/microsome fractions, the post-mitochondrial supernatant was passed through stepwise sucrose gradients (1.5–0.25 M) and sample-containing tube was centrifuged at $280,000 \times g$ for 1 h. For quality assessment of mitochondrion fraction, succinic dehydrogenase activity assay was carried out as described previously (Jones et al. 2013). For ER/microsome fraction, cytochrome p450 reductase assay was carried out for quality assessment as described previously (Yim et al. 2005).

Statistical analysis

The data was analyzed statistically using SPSS 13.0. The results were analyzed using one-way ANOVA to determine the significance of the mean between groups. Values of $P < 0.005$ were considered to be significant. The means of the data are presented together with standard error of mean (SEM) from multiple independent experiments.

Results and discussion

It has been a well-known fact that microenvironment in exponentially growing cancer is generally acidic as compared to normal tissue and low extracellular pH has been documented to induce coordinate regulation of gene expression in cells (Duggan et al. 2006).

Acidic environment-induced apoptosis

Anti-proliferative effect of acidic pH on Raji cells was studied by MTT assay. Cells were grown in media of pH 6.8 and 5.8, and cell viability was analyzed. After 12 h of incubation, no significant variation was observed at different pH. After 24 h of incubation at pH 6.8, no variation in cell viability was observed while nearly 89 % cells were viable at pH 5.8 after 24 h. A drastic change was observed after 48 h of incubation. At pH 5.8, the survival of cells decreased to nearly 2.5-fold when compared with cell viability at pH 6.8. To examine the effect of acidic stress treatment on cancer cells, Raji cells were

Fig. 2 Increase in intracellular calcium at acidic pH. Raji cells were cultured in normal (pH 7.3) and acidic media pH 6.8 and pH 5.8 for 48 h followed by incubation with the fluorescent dye Fluo 3-AM. Representative FACS analysis histograms of Fluo 3-AM stained Raji

cells in control and after acidic stress treatment. Five thousand events were counted. Increase in calcium at pH 5.8 was found statically significant (p value < 0.005) with respect to control. Cells at physiological pH (pH 7.3) were taken as control

cultured in different pH media (7.3, 6.8, 6.3, and 5.8) for 48 h and analyzed for apoptosis by DNA laddering assay. In comparison to control (pH 7.3) and cells grown in medium of pH 6.8, the DNA of Raji cells grown in pH 5.8 medium demonstrated markedly increased fragmented DNA (Fig. 1). These results indicate the involvement of apoptosis in low (acidic)-pH-mediated cell cytotoxicity in Raji cells. These results were in agreement with the earlier studies which strongly suggested that acidic stress can induce DNA damage and apoptosis (Jolly et al. 2004). Flow cytometric evaluation of cell morphology at low pH showed a shift in cellular granularity, which further suggested unhealthy or cells prone to apoptosis at the acidic pH as compared to control (pH 7.3) (Fig. 2).

Acidic stress-affected calcium homeostasis

To interrogate the role of acidic stress on Ca^{2+} homeostasis, we selectively examined cytosolic Ca^{2+} in Raji cells in normal and acidic stress conditions. The increase of ER membrane permeability and calcium release from ER are related with the induction of apoptosis in tumor cells (Boya et al. 2002). In comparison to control (pH 7.3), approximately 8-fold enhanced cytosolic Ca^{2+} was detected in Raji cells when pH of the growth medium was shifted to 5.8 (Fig. 2). Thus, acid stress-mediated dysregulation of Ca^{2+} homeostasis might indicate ER stress which in turn triggers apoptosis.

Acidic stress-induced endoplasmic reticulum stress

Considering the enhanced cytosolic Ca^{2+} after acidic stress, we further studied the effect of acidic stress on endoplasmic reticulum, the major cellular store of calcium. Factors and conditions that disrupt protein folding in the endoplasmic

Fig. 3 Effect of acidic stress on *XBP1* splicing, CHOP, and GRP78 in Raji cells. Raji cells were grown at pH 7.3, pH 6.8, and pH 5.8 (acidic stress) for 48 h. Total RNA was extracted from the cells, and the mRNA of *XBP1* was detected and analyzed by RT-PCR. The RT-PCR products of *XBP1* were analyzed on 12.5 % PAGE, and GRP78 and CHOP were analyzed on 2 % agarose gel. β -Actin was used as internal control. Cells grown at physiological pH 7.3 were taken as control

reticulum, such as a chemical, nutrient, and environmental stress, activate stress signaling pathway jointly known as unfolded protein response (UPR). UPR activation ultimately moves the cell physiology toward cell-cycle arrest and the induction of apoptosis. Endoplasmic reticulum stress induces activation of the IRE1, an endonuclease that causes the unconventional splicing of mRNA encoding a UPR-specific transcription factor, called X-box binding protein 1 (XBP1) (Schadewijk et al. 2012). UPR is mediated by upregulation of chaperones such as GRP78, and apoptotic pathways are activated and mediated by the induction of CHOP. Induction of stress response genes *XBP1*, *GRP78*, and *CHOP* in response to growth of cells in acidic environment was studied by RT-PCR. To evaluate the induction of IRE1 α /XBP1 pathway in the cells grown under acidic environment, *XBP1* splicing was examined by RT-PCR. *XBP1* mRNA splicing, indicative of ER stress, was detected in the cells grown at pH 5.8 (Fig. 3). In contrast, *XBP1* splicing was not induced in the cells grown at pH 6.8 and 7.3. Similarly, upregulation of GRP78 and CHOP mRNA in cells exposed to acidic pH provides further evidence of generation of ER stress in these cells. Thus, the exposure of Raji cells to

Fig. 4 *Bax* induction and translocation to mitochondrion by low pH. **a** Raji cells were incubated in acidic environment pH 6.8 and pH 5.8 for 48 h. Expression analysis was carried out by quantitative real-time PCR. Real-time PCR was carried out using gene-specific primers, and specificity of product was confirmed by melting curve analysis. The data was normalized to β -actin. **b** Subcellular fractionation was performed to obtain the fractions for cytosol, mitochondria, and ER/microsome followed by western blot analysis using anti Bax-specific monoclonal antibody. Under acidic environment, a significant increase in Bax expression at pH 5.8 was observed in mitochondrion fraction while in contrast to it, Bax expression decreased in endoplasmic fractions. Cells grown at physiological pH (pH 7.3) were taken as control

extracellular acidic environment resulted in activation of endoplasmic reticulum stress response.

Acidic stress-mediated cell death associated with Bax translocation to mitochondrion

Bax, a proapoptotic Bcl-2 family member, is involved in mitochondrial apoptotic signaling pathway. Bax is also known to be induced by p53, and Bax redistributes both to mitochondria and ER upon apoptosis induction (Smith and Deshmukh 2007). Therefore, the expression of *Bax* after exposure of cells

to acidic environment was evaluated by quantitative RT-PCR. The expression of *Bax* was upregulated (4.2-fold) in cells grown under acidic stress, i.e., pH 5.8 as compared to control (Fig. 4). These results suggested that acidic stress-mediated induction of apoptosis is associated with Bax. Further, to confirm the role of Bax in apoptosis, cytosolic, endoplasmic reticulum, and mitochondrion fractions were isolated and analyzed for Bax distribution. In cytosolic fractions, no significant changes in the level of Bax were observed while in endoplasmic reticulum fraction, a significant decrease was observed at pH 5.8. In contrast to it, a significant increase in Bax

Fig. 5 Acidic stress treatment leads to enhanced cytochrome c release and caspase-3 activation. **a** Representative FACS analysis histograms of anti-cytochrome c stained Raji cells after acidic stress. Bar graph showing cytochrome c expression after acidic stress treatment expressed as mean fluorescence intensity. **b** Representative FACS analysis histograms of anti-active caspase-3 stained Raji cells after acidic stress. Bar graph showing

caspase-3 expressions after acidic stress treatment expressed as mean fluorescence intensity. A significant (P value <0.005) upregulation in active caspase-3 was observed with respect to control. Cells grown at physiological pH (pH 7.3) were taken as control. Five thousand events were counted per tube. **c** Illustrated PARP cleavage leads to generation of 85-kDa fragments as compare to control

level was observed in mitochondrion fraction at pH 5.8 as compared to control (pH 7.3). These results are in agreement with earlier studies, which strongly suggested that endoplasmic reticulum stress causes robust activation, translocation, and N-terminal exposure of Bax in the mitochondria (Zhang and Armstrong 2007).

Acidic stress caused cytochrome c release, enhanced active caspase-3 expression, and PARP cleavage

Both Bax and calcium have been documented to alter mitochondrial permeability. To reveal the role of mitochondria in acidic stress-induced apoptosis, the status of cytosolic cytochrome c in normal and cells grown in acidic medium was analyzed (Fig. 5a). Culturing of Raji cells in acidic environment (pH 5.8) leads to nearly four times higher release of cytochrome c when compared to control. Cytochrome c is an intermediate in apoptosis. A major consequence of cytochrome c release is the induction of caspase cascade mediated apoptosis induction. To verify caspase induction, the expression of active caspase-3, which is a downstream target of caspase-9, was analyzed by flow cytometry (Fig. 5b). Raji cells, cultured at acidic pH 5.8, demonstrated nearly 2.3-fold increase in expression of active caspase-3 as compared to control. Incubation of Raji cells in acidic media resulted in cleavage of poly(ADP-ribose) polymerase (PARP) to 85-kDa fragment. PARP cleavage was significant in pH 6.8 medium, and it further increased in pH 5.8 medium (Fig. 5c). These results suggested that acidic stress-induced apoptosis

was accompanied by cytochrome c release which in turn leads to the induction of caspase-3 mediated apoptotic pathway.

Acidic stress mediated ROS independent apoptosis

In order to investigate the effect of acidic pH medium on ROS-mediated induction of apoptosis, we performed flow cytometric analysis on Raji cells after treatment at pH 6.8 and pH 5.8. We did not observe any significant alteration in ROS after acidic stress treatment (Fig. 6) after 48 and 24 h (supplementary Fig. 1). The result suggests that ROS is not involved in inducing apoptosis in Raji cells in acidic growth conditions.

Acidic stress caused upregulation of *TP53*, *p21*, and nuclear localization of NF- κ B

Recently, studies have suggested that endoplasmic reticulum stress involves the key role of NF- κ B in regulation of p53 and its association with endoplasmic reticulum (Lin et al. 2012; Yang et al. 2013). Therefore, the effect of acidic stress on localization of NF- κ B along with expression of *TP53* and its downstream effector *p21* was investigated. The p21 (CIP1/WAF1) protein that is upregulated by p53 in response to various stimuli binds to and inhibits the activity of cyclin-CDK2 or CDK1 complexes and thus functions as a regulator of cell cycle progression. In response to extracellular acidic stress, the level of *TP53* mRNA was enhanced up to about 7-fold and *p21* mRNA was approximately 14-fold higher as compared with the control (Fig. 7a). These findings are in

Fig. 6 Low pH caused ROS independent cell death. Raji cells exposed to acidic stress were incubated with DCFH-DA after and 48 h followed by flow cytometry analysis. Cells grown at physiological pH (pH 7.3) were taken as control. Acidic stress-mediated induction of apoptosis was found to be independent of ROS

Fig. 7 Acidic environment upregulated mRNA level of *TP53* and *p21* and elevated the expression of NF- κ B, p53, and p21 in Raji cells. **a** Quantitative real-time PCR-based analysis of *TP53* and *p21* expression. RNA was isolated from control and acidic stress (pH 5.8 and pH 6.8) exposed Raji cells. Real-time PCR was carried out using gene-specific primers, and specificity of product was confirmed by melting curve analysis. A significant (P value <0.005) increase in p21 mRNA was found at

pH 6.8 and pH 5.8 with respect to control. **b** Representative FACS analysis histograms of anti-p21 stained Raji cells after acidic stress. Bar graph showing p21 expression after acidic stress treatment expressed as mean fluorescence intensity. **c** Induction of p53 and nuclear localization of NF- κ B by acidic environment in Raji cells. The data was normalized to β -actin for real-time analysis and GAPDH for Western blot analysis. Cells at pH (pH 7.3) were taken as control

accordance with the earlier reports which suggested the activation of *TP53* and *p21* in acidic environment (Williams et al. 1999). The elevated level of p53 and *p21* transcripts correlated with the elevated expression of p53 and p21 proteins in acidic stress condition (Fig. 7c, b). Although the induction of p53 in response to acidic environment exposure is correlated with upregulation of *p21* expression, it is not conclusive that mutated p53 in Raji cells can upregulate *p21* expression. It is possible that p53

in Raji cell line which harbors two p53 alleles each having a point mutation resulting in changed amino acids (Duthu et al. 1992) retains some of its wild-type function to regulate *p21* expression. Further, NF- κ B translocation to nucleus in response to acidic environment (Fig. 7c) also indicated that p53 might be regulated by NF- κ B in Raji cells. It suggested that under acidic pH, the cumulative effect of calcium and Bax induction along with p53 induction might contribute to apoptosis.

Fig. 8 Proposed hypothesis or pathway

Conclusion

The findings from the present study suggest that acidic stress causes apoptosis of Raji cells via mitochondrion-dependent and ER stress-triggered signaling pathway. This is evident from acidic stress-mediated enhancement of cytosolic Ca^{+2} , stimulation of *XBPI* splicing, induction of *Bax*, and release of cytochrome c in response to acidic stress (Fig. 8). Therefore, it is proposed that acidic stress could activate distinct apoptotic events, which involve ER stress generation and mitochondrial membrane permeability transition. To the best of our knowledge, it is the first report showing induction of endoplasmic reticulum stress under acidic stress in Raji cells

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References

- Bhat V, Kuroski D, Kuroski D, McDonald CB, Mikles DC, Deegan BJ, Seldeen KL, Lednev IK, Farooq A (2012) Acidic pH promotes oligomerization and membrane insertion of the BclXL apoptotic repressor. *Arch Biochem Biophys* 528:32–44
- Boya P, Cohen I, Zamzami N, Vieira HL, Kroemer G (2002) Endoplasmic reticulum stress-induced cell death requires mitochondrial membrane permeabilization. *Cell Death Differ* 9:465–467
- Chen KH, Tung PY, Wu JC, Chen Y, Chen PC, Huang SH, Wang SM (2008) An acidic extracellular pH induces Src kinase-dependent loss of beta-catenin from the adherens junction. *Cancer Lett* 267:37–48
- Chu G, Dewey WC (1988) The role of low intracellular or extracellular pH in sensitization to hyperthermia. *Radiat Res* 114:154–167
- Duggan SP, Gallagher WM, Fox EJP, Abdel-Latif MM, Reynolds JV, Kelleher D (2006) Low pH induces co-ordinate regulation of gene expression in oesophageal cells. *Carcinogenesis* 27:319–327
- Duthu A, Debuire B, Romano J, Ehrhart JC, Fiscella M, May E, Appella E, May P (1992) p53 mutations in Raji cells: characterization and localization relative to other Burkitt lymphomas. *Oncogene* 11: 2161–7
- Fields WR, Leonard RM, Odom PS, Nordskog BK, Ogden MW, Doolittle DJ (2005) Gene expression in normal human bronchial epithelial (NHBE) cells following *in vitro* exposure to cigarette smoke condensate. *Toxicol Sci* 86:84–91
- Fukamachi T, Chiba Y, Wanga X, Saito H, Tagawa M, Kobayashi H (2010) Tumor specific low pH environments enhance the cytotoxicity of lovastatin and cantharidin. *Cancer Lett* 297:182–9
- Fukumura D, Xu L, Chen Y, Gohongi T, Seed B, Jain RK (2001) Hypoxia and acidosis independently up-regulate vascular endothelial growth factor transcription in brain tumors *in vivo*. *Cancer Res* 61:6020–6024
- Gong J, Traganos F, Darzynkiewicz Z (1994) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal Biochem* 218:314–319
- Haveman J (1979) The pH of the cytoplasm as an important factor in the survival of vitro cultured malignant cells after hyperthermia, effects of carbonyl cyanide-3-chlorophylhydrazide. *Eur J Cancer* 15:1281–1288
- Heckenkamp J, Leszczynski D, Schiereck J, Kung J, La Muraglia GM (1999) Different effects of photodynamic therapy and gamma irradiation on vascular smooth muscle cells and matrix: implications for inhibiting restenosis. *Arterioscler Thromb Vasc Biol* 19:2154–2161
- Hjelmeland AB, Wu Q, Heddleston JM, Choudhary GS, MacSwords J, Lathia JD, McLendon R, Lindner D, Sloan A, Rich JN (2011) Acidic stress promotes a glioma stem cell phenotype. *Cell Death Differ* 18:829–840
- Jha AK, Nikbakht M, Parashar G, Shrivastava A, Capalash N, Kaur J (2010) Reversal of hypermethylation and reactivation of the RARβ2 gene by natural compounds in cervical cancer cell lines. *Folia Biol* 56:195–2010
- Jolly AJ, Wild CP, Hardie LJ (2004) Acid and bile salts induce DNA damage in human oesophageal cell lines. *Mutagenesis* 19:319–324
- Jones AJY, Judy Hirst JA (2013) A spectrophotometric coupled enzyme assay to measure the activity of succinate dehydrogenase. *Anal Biochem* 442:19–23
- Kato Y, Ozono S, Shuin T, Miyazaki K (1996) Slow induction of gelatinase B mRNA by acidic culture conditions in mouse metastatic melanoma cells. *Cell Biol Int* 20:375–377
- Kato Y, Lambert CA, Colige AC, Mineur P, Noel A, Franken F, Foidart JM, Baba M, Hata R, Miyazaki K, Tsukuda M (2005) Acidic extracellular pH induces matrix metalloproteinase-9 expression in mouse metastatic melanoma cells through the phospholipase D-mitogen-activated protein kinase signaling. *J Biol Chem* 280:10938–10944

- Lin WC, Chuang YC, Chang YS, Lai MD, Teng YN, Su IJ, Wang CCC, Lee KH, Hung JH (2012) Endoplasmic reticulum stress stimulates p53 expression through NF- κ B activation. *PLoS ONE* 7(7):e39120
- Mortensen BT, Jensen PO, Helledie N, Iversen PO, Ralekeir E, Larsen JK, Madsen MT (1998) Changing bone marrow microenvironment during development of acute myeloid leukemia in rats. *Br J Haemat* 102:458–464
- Park HJ, Lyons JC, Ohtsubo T, Song CW (1999) Acidic environment causes apoptosis by increasing caspase activity. *Br J Cancer* 80: 1892–1897
- Reichert M, Steinbach JP, Supra P, Weller M (2002) Modulation of growth and radiochemosensitivity of human malignant glioma cells by acidosis. *Cancer* 95:1113–9
- Rofstad EK, Mathiesen B, Kindem K, Galappathi K (2006) Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer Res* 66:6699–6707
- Schadewijk AV, Emily FA, Wout V, Stolk J, Hiemstra PS (2012) A quantitative method for detection of spliced X-box binding protein-1 (XBP1) mRNA as a measure of endoplasmic reticulum (ER) stress. *Cell Stress* 17:275–279
- Schornack PA, Gillies RJ (2003) Contributions of cell metabolism and H⁺ diffusion to the acidic pH of tumors. *Neoplasia* 5:135–145
- Shamim U, Hanif S, Albanyan A, Beck FW, Bao B, Wang Z, Banerjee S, Sarkar FH, Mohammad RM, Hadi SM, Azmi AS (2012) Resveratrol-induced apoptosis is enhanced in low pH environments associated with cancer. *J Cell Physiol* 227:1493–1500
- Sharma L, Kaur J, Shukla G (2012) Role of oxidative stress and apoptosis in the placental pathology of plasmodium berghei infected mice. *PLoS ONE* 7(3):e32694
- Smith MI, Deshmukh M (2007) Endoplasmic reticulum stress-induced apoptosis requires bax for commitment and Apaf-1 for execution in primary neurons. *Cell Death Differ* 14:1011–1019
- Song CW, Griffin R, Park HJ (2006) Influence of tumor pH on therapeutic response. *Cancer Drug Discover and Develop*, pp 21–42
- Tannock IF, Rotin D (1989) Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res* 49:4373–4384
- Vaupel P, Kallinowski F, Okunieff P (1989) Blood flow, oxygen and nutrient supply and metabolic microenvironment of human tumors: a review. *Cancer Res* 49:6449–6465
- Wang FM, Galson DL, David Roodman G, Hongjiao Ouyang GD (2011) Resveratrol triggers the pro-apoptotic endoplasmic reticulum stress response and represses the pro-survival XBP1 signaling in human multiple myeloma cells. *Exp Hematol* 39: 999–1006
- Williams AC, Collard TJ, Paraskeva C (1999) An acidic environment leads to p53 dependent induction of apoptosis in human adenoma and carcinoma cell lines: implications for clonal selection during colorectal carcinogenesis. *Oncogene* 18:3199–3204
- Xu L, Fidler IJ (2000) Acidic pH-induced elevation in interleukin 8 expression by human ovarian carcinoma Cells. *Cancer Res* 60:4610–4616
- Xu L, Fukumura D, Jain RK (2002) Acidic extracellular pH induces vascular endothelial growth factor (VEGF) in human glioblastoma cells via ERK1/2 MAPK signaling pathway: mechanism of low pH-induced VEGF. *J Biol Chem* 277:11368–11374
- Yang PM, Lin YT, Shun CT, Lin SH, Wei TT, Chuang SH, Wu MS, Chen CC (2013) Zebularine inhibits tumorigenesis and stemness of colorectal cancer via p53-dependent endoplasmic reticulum stress. *Sci Rep* 3:3219
- Yim SK, Yun CH, Ahn T, Jung HC, Pan JG (2005) A continuous spectrophotometric assay for NADPH-cytochrome P450 reductase activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. *J Biochem Mol Biol* 38:366–369
- Zhang D, Armstrong JS (2007) Bax and the mitochondrial permeability transition cooperate in the release of cytochrome c during endoplasmic reticulum-stress-induced apoptosis. *Cell Death Differ* 14:703–715
- Zhu S, Wang Y, Jin J, Guan C, Li M, Xi C, Ouyang Z, Chen M, Qiu Y, Huang M a, Huang Z (2012) Endoplasmic reticulum stress mediates aristolochic acid I-induced apoptosis in human renal proximal tubular epithelial cells, *Toxicology in Vitro*.; xxx: xxx–xxx