In Non-neoplastic Barrett’s Epithelial Cells, Acid Exerts Early Antiproliferative Effects through Activation of the Chk2 Pathway

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Abstract
Acid exerts pro-proliferative effects in Barrett’s-associated esophageal adenocarcinoma cells. In non-neoplastic Barrett’s epithelial (BAR-T) cells, in contrast, we have shown that acid exposure has antiproliferative effects. To explore our hypothesis that the acid-induced, antiproliferative effects are mediated by alterations in the proteins that regulate the G1-S cell cycle checkpoint, we exposed non-neoplastic Barrett’s cells to acidic media (pH 4.0) and analyzed G1-S checkpoint proteins’ expression, phosphorylation, and activity levels by Western blot. We studied acid effects on growth (by cell counts), proliferation (by flow cytometry and bromodeoxyuridine incorporation), cell viability (by trypan blue staining), and apoptosis (by annexin V staining), and we used caffeine and small interfering RNA to assess the effects of checkpoint kinase 2 (Chk2) inhibition on G1-S progression. Acid exposure significantly decreased cell numbers without affecting cell viability and with only a slight increase in apoptosis. Within 2 h of acid exposure, there was a delay in progression through the G1-S checkpoint that was associated with increased phosphorylation of Chk2, decreased levels of Cdc25A, and decreased activity of cyclin E–cyclin-dependent kinase 2; by 4 h, a continued delay at G1-S was associated with increased expression of p53 and p21. Caffeine and Chk2 siRNA abolished the acid-induced G1-S delay at 2 but not at 4 h. We conclude that acid exposure in non-neoplastic BAR-T cells causes early antiproliferative effects that are mediated by the activation of Chk2. Thus, we have elucidated a mechanism whereby acid can exert disparate effects on proliferation in neoplastic and non-neoplastic BAR-T cells. [Cancer Res 2007;67(18):8580–7]

Introduction
In the last three decades, the incidence of esophageal adenocarcinoma has increased ~600% in the United States (1, 2). The major risk factors for this cancer are gastroesophageal reflux disease (GERD) and Barrett’s esophagus, the condition in which a metaplastic, intestinal-type epithelium replaces esophageal squamous epithelium that has been damaged by GERD. This metaplastic epithelium is predisposed to malignancy, and most esophageal adenocarcinomas are thought to arise from Barrett’s esophagus (3, 4).

It has been proposed that acid reflux promotes the progression of Barrett’s metaplasia to esophageal adenocarcinoma, based largely on the observations that acid exposure can induce pro-proliferative effects in cultures of Barrett’s biopsy specimens (explants) and in cultures of Barrett’s-associated esophageal adenocarcinoma cells (5–7). Those in vitro model systems are not ideal for the study of the early carcinogenetic events in Barrett’s epithelial (BAR-T) cells, however. Explants of Barrett’s mucosa comprise both epithelial and non-epithelial cell types (e.g., inflammatory and stromal cells), and it is difficult to ascertain which of the diverse cell types proliferate in response to acid. Barrett’s-associated adenocarcinoma cells have numerous uncharacterized genetic abnormalities, and acid-induced effects in those cells may not reflect the effects of acid on non-neoplastic BAR-T cells.

To develop an in vitro model for Barrett’s esophagus that avoids the pitfalls of explants and adenocarcinoma cell cultures, we have established cultures of BAR-T cells that are immortalized, but not transformed, through the forced expression of telomerase (8). We have shown that these cells retain a diploid karyotype, express differentiation markers typical of benign Barrett’s epithelium, and exhibit phenotypic features characteristic of non-neoplastic cells, including contact inhibition, anchorage-dependent growth, and the expression of functional cell cycle checkpoint proteins (8). Using cultures of BAR-T cells, we have found that acid exerts antiproliferative effects (9).

Cellular proliferation is a manifestation of passage through the cell cycle, which is regulated at various checkpoints. Genomic damage can activate cell cycle checkpoints to halt progression through the cycle, enabling the cell to repair its DNA damage or, if the damage is extensive and irreparable, to trigger cellular destruction by apoptosis (10). Many crucial cell fate decisions are made at the key checkpoint that regulates the transition from G1 to S phase. Therefore, a potential mechanism whereby acid exposure could influence proliferation and apoptosis in BAR-T cells is by affecting the proteins that regulate the G1-S cell cycle checkpoint.

A number of proteins are known to influence passage through the G1-S cell cycle checkpoint. For example, Cdc25A can activate the cyclin E–cyclin-dependent kinase 2 (Cdk2) complex, which enables progression through the G1-S checkpoint (10, 11). In contrast, progression through the checkpoint can be blocked by activating the p53 pathway, which results in the transcription of genes like p21 whose products inhibit the actions of cyclin-Cdk complexes. G1-S progression can also be delayed by activating the checkpoint kinases 1 and 2 (Chk1 and Chk2), which mediate the degradation of Cdc25A (10). To investigate mechanisms whereby acid reflux might influence proliferation and apoptosis in Barrett’s...
esophagus, we exposed our BAR-T cells to acid and studied the resulting effects on cell growth and on proteins that regulate the G1-S cell cycle checkpoint.

Materials and Methods

Cell culture. We used three telomerase-immortalized, nontransformed, BAR-T cell lines (BAR-T, BAR-T 9, and BAR-T 10) created from endoscopic biopsy specimens of non-dysplastic Barrett's specialized intestinal metaplasia taken from three patients with long-segment (>3-cm) Barrett's esophagus. The cells were cultured in growth media consisting of supplemented keratinocyte basal media, KBM-2 (Cambrex Biologicals) and maintained as previously described (8, 12). For individual experiments, cells were equally seeded into collagen IV–coated wells (BD Biosciences) and maintained in growth media. We selected to use the BAR-T line for all experiments unless otherwise indicated because this line has been extensively characterized by our laboratory (8, 9, 13).

Acid exposure. For individual experiments, the cells were cultured either in neutral full-growth media (pH 7.2) or in acidic full-growth media (brought to a pH of 4.0 with 1 mol/L HCl). The neutral or acidic media was added for 10 min (unless otherwise indicated) to equally seeded wells of Barrett's cells and then removed and replaced with neutral pH media for the remainder of the experiment.

Determination of cell number, cell viability, apoptosis, and cell cycle distribution. At 24 h after acid exposure, cell number was determined by cell counts with a Z1 particle counter, and cell viability was assessed by trypan blue staining. Apoptosis rates were assessed by analysis of annexin V (BD Biosciences) and propidium iodide (PI; Sigma) staining by flow cytometry (9); cells staining only with annexin V were considered to be apoptotic. Cellular morphology was documented using the Metamorph imaging system (Universal Imaging Corporation); UV-B irradiated cells were used as a positive control (13). For cell-cycle analyses, Barrett's cells were synchronized for 24 h in KBM-2 lacking growth factor and serum supplemented and then allowed to recover for 14 h in the neutral pH growth media before a 10-min acid exposure. Cells were collected just before release in neutral pH growth media (time 0) and at 2 and 4 h after the acid exposure or neutral pH media change (time points that correspond to 16 and 18 h after release into growth media, respectively). Before analysis by flow cytometry, cells were stained with 50 μg/mL of PI. Cell proliferation was also assessed using the Cell Proliferation ELISA (Roche) per manufacturer's instructions.

Western blotting. Cells were exposed to 10 min of acid or neutral pH media change, and lysates were collected immediately thereafter (time 0) and at various other time points using Cell Signaling lysis buffer supplemented with 1 mmol/L phenylmethylsulfonyl fluoride (Sigma) and then sonicated briefly. Equal amounts of protein were separated by SDS-PAGE; protein concentrations were determined using the BCA-200 Protein Assay Kit (Pierce). After separation and transfer to polyvinylidene difluoride (PVDF) membranes, the membranes were incubated with 1:1,000 dilutions of mouse monoclonal anti-human p53, Cdk4 (Calbiochem), or Cdk2A (Santa Cruz Biotechnology), 1:500 dilutions of mouse monoclonal anti-human p21 (Oncogene), 1:1,000 dilutions of rabbit polyclonal anti-human p53 (Ser15), total Chk1, phospho-Chk1 (Ser17 and Ser345), total Chk2, or phospho-Chk2 (Thr68; Cell Signaling). For detection of phospho-Cdk2 (Thr14/Tyr15; Santa Cruz Biotechnology), equal amounts of protein were immunoprecipitated for 3 h at 4°C with saturating amounts of anti-phospho-Cdk2 (Thr14/Tyr15); followed by overnight incubation with protein A–sepharose beads at 4°C. Sepharose-bound immunoprecipitated proteins were then washed thrice with ice-cold lysis buffer, followed by separation and transfer to PVDF membranes. The membranes were incubated with 1:1,000 dilutions of rabbit polyclonal anti-human total Cdk2 (Santa Cruz Biotechnology). Anti-mouse (1:5,000) or anti-rabbit (1:2,000) horseradish peroxidase secondary antibody (Santa Cruz Biotechnology) was used, and chemiluminescence was determined using the Super Signal West Dura detection system (Pierce). All studies were done in duplicate. Membranes were then stripped using Restore Stripping Buffer (Pierce) and reprobed with β-actin (Sigma) to confirm equal loading. Relative band intensities were determined by densitometry using the MultiAnalyst software package (Bio-Rad).

Cyclin-Cdk assays. For cyclin E–Cdk2 activity assays, cells were exposed to 10 min of acid, and lysates were collected immediately thereafter (time 0) and at various other time points. Lysates were clarified by centrifugation at 14,000 × g for 10 min at 4°C. Following a bicinchoninic acid protein assay, equal amounts of protein were immunoprecipitated overnight at 4°C with saturating amounts of agarose-conjugated anti–cyclin E (Santa Cruz Biotechnology). Immunoprecipitated proteins were then washed thrice with ice-cold lysis buffer and then twice with ice-cold kinase buffer (Cell Signaling). Cyclin E–Cdk2 kinase activity was determined using histone H1 as the substrate for in vitro phosphorylation. The agarose bound with cyclin E–Cdk2 complexes was resuspended in 10 μL of kinase buffer containing 0.25 μg/μL histone H1 (Roche), 40 μmol/L ATP, and 0.5 μCi/μL [γ-32P] ATP (Amersham Biosciences) and incubated at 30°C for 30 min. The kinase reaction was stopped by adding 3 μL of 4× electrophoresis sample buffer, followed by boiling for 3 min at 95°C. The reaction products were then electrophoresed on 12% polyacrylamide gels and visualized by autoradiography of the dried slab gels. For cyclin D1–Cdk4 immunoprecipitation and activity assays, the same protocol was followed except that we used agarose-conjugated anti-cyclin D1 (Santa Cruz Biotechnology), 40 μmol/L ATP, and the Rb-C fusion protein (2 μg/20 μL reaction), containing residues 701 to 928, which include the sites of phosphorylation by Cdk4 as the substrate (14–16). Western blot was done using 1:1,000 dilutions of rabbit polyclonal anti-human-phospho-Rb (Ser780; Cell Signaling).

Chk inhibitors. The Chk2 pathway was blocked using 10 mmol/L caffeine (Sigma), an inhibitor of the ATM/ATR kinases, which are immediately upstream of Chk2, and using SMARTpool siRNA duplexes against Chk2 (Upstate Biotechnology; ref. 17). Equally seeded wells of Barrett's cells were transiently transfected with 200 μmol/L siRNA using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. As controls, cells were transfected with nonspecific control SMARTpool siRNA oligonucleotides (Upstate) or treated with LipofectAMINE 2000 reagent alone. Cells were pretreated with caffeine for 1 h and then exposed to acidic media containing caffeine for 10 min. Forty-eight hours after transfection with Chk2 siRNA, cells were exposed to acid for 10 min.

Statistical analyses. Quantitative data are expressed as the mean ± SE. Statistical analysis was done using either the unpaired Student's t test or an ANOVA in combination with the Student-Newman-Keuls multiple-comparison test with the Instat for Windows statistical software package (GraphPad Software). P values <0.05 were considered significant for all analyses.

Results

Acid exposure decreases BAR-T cell numbers at 24 h. We have previously found that two, 3-min acid exposures given 60 min apart decreases the cell number of BAR-T cells (9). To determine the effect of a single acid exposure, BAR-T cells were given a single 3- or 10-min acid exposure, and cell numbers were determined. These durations were chosen to simulate typical, physiologic episodes of gastroesophageal reflux (18). Compared with no acid treatment, a single acid exposure given for either 3 or 10 min significantly decreased cell numbers at 24 h (Fig. 1A). After reviewing these data, we selected to use the 10-min acid exposure for all further experiments. We then determined the effect of a single, 10-min acid exposure in two additional telomerase-immortalized metastatic Barrett’s cell lines (BAR-T9 and BAR-T10). Compared with no acid treatment, a single 10-min acid exposure significantly decreased cell numbers at 24 h in all three Barrett’s cell lines (Fig. 1B).

Acid exposure does not affect cell viability, but causes a small increase in apoptosis. To explore the mechanisms underlying the acid-induced decrease in cell number, we studied...
cell viability and apoptosis. Trypan blue staining revealed no significant difference in cell viability between acid-exposed and nonexposed cells at 24 h (data not shown). We also found no difference in morphology between acid-exposed and nonexposed cells (Fig. 2A). In contrast, UV-B irradiation resulted in small and shrunken cells, phenotypic changes suggestive of apoptosis (Fig. 2A). Using annexin V and PI staining, however, we found a small but statistically significant increase in the fraction of apoptotic cells in the acid-exposed group (3.8 ± 0.37% SE) compared with untreated control cells (2.7 ± 0.14% SE; Fig. 2B).

Acid exposure decreases proliferation by delaying G_1 to S phase transition. Having found that neither decreased cell viability nor increased apoptosis could account for the decreased cell number following acid exposure, we next determined whether acid delayed progression from G_1 to S phase. Cells were synchronized for 24 h in KBM-2 lacking growth factor and serum supplementation, followed by release into growth media. In preliminary time course experiments, we determined that movement from G_1 into S occurred between 12 and 18 h after release into growth media (data not shown). Therefore, BAR-T cells were released into growth media for 14 h and then exposed to acid for 10 min. Movement from G_1 to S phase was analyzed by flow cytometry 2 and 4 h after acid exposure (time points that correspond to 16 and 18 h after release into the growth media, respectively). At 2 h, the fraction of cells in G_1 remained higher in the acid-treated group, although this difference was not statistically significant (P = 0.11; Fig. 3A). Moreover, the fraction of cells in the S phase at this 2-h time point was significantly lower in the acid-treated group (Fig. 3A). The acid-induced decrease in cells entering the S phase at 2 h was confirmed by bromodeoxyuridine (BrdUrd) incorporation (Fig. 3B). By 4 h, the fraction of cells remaining in G_1 was significantly higher, and the fraction of cells in S remained significantly lower in the acid-exposed cells (Fig. 3A). There was no significant difference in the fraction of cells in G_2 at either time point between acid-exposed and nonexposed cells (data not shown). Compared with the 2-h time point, furthermore, the 4-h time point had a significantly smaller fraction of cells in G_1 [76.6 ± 0.3% versus 89.3 ± 2.8%; P = 0.01] and a significantly larger fraction of cells in the S phase (229.6 ± 1.5% versus 181.0 ± 1.8%; P < 0.0001), suggesting that the acid caused a delay in progression from G_1 to S rather than a complete G_1 arrest (Fig. 3A).

Acid exposure increases expression of p53, phospho-p53 Ser^{15}, and p21. Because p53 and its downstream transcriptional target, p21, are known to play key regulatory roles in the G_1-S cell cycle checkpoint, we investigated the effect of acid exposure on the expression of p53 and p21 proteins (19). Furthermore, because site-specific phosphorylation of p53 can determine whether the protein mediates transcriptional activity (Ser^{15} phosphorylation) or apoptotic activity (Ser^{46} phosphorylation), these two phosphorylated forms of p53 were also investigated (20, 21). Total p53, phospho-p53 (Ser^{15} and Ser^{46}), and p21 protein expression were determined by Western blot immediately after acid exposure (time 0) and at 15 and 30 min, 1, 2, 4, and 6 h later. Total p53 expression increased beginning at 1 h and remained elevated at 6 h following acid exposure (Fig. 4). Phospho-p53 Ser^{15} also increased beginning at 1 h, with maximal expression by 4 h (Fig. 4). There was no expression of phospho-p53 Ser^{46} at any of the time points (data not shown). Increased p21 expression was not apparent until 4 h after acid exposure (Fig. 4). Thus, acid exposure increases the expression of p53, phospho-p53 Ser^{15}, and p21 but not phospho-p53 Ser^{46} in BAR-T cells. We noted that although the increase in p21 expression did not appear until 4 h after the acid exposure, the delay in G_1-S transition was evident by 2 h. This suggests that induction of p21 by p53 was not responsible for the early, acid-induced delay in G_1-S transition (Figs. 3A and 4). Therefore, we next investigated expression levels of Cdc25A, a protein known to regulate a rapid G_1-S checkpoint response.

Acid exposure rapidly decreases the levels of Cdc25A protein and cyclin E activity. G_1-S regulation has been observed in cells that lack p53 and p21 (22, 23). Cdc25A may serve as an early regulator of that checkpoint. Cdc25A activates cyclin-Cdk complexes by dephosphorylating the Cdk, and if Cdc25A levels decrease, progression through the G_1-S checkpoint is halted rapidly (10, 24). We investigated whether acid exposure altered expression levels of Cdc25A, phospho-Cdk2 (Thr^{147/Tyr^{157}}), and activities of the Cdk4/6–cyclin D1 and Cdk2–cyclin E complexes. Western blots showed that Cdc25A expression levels decreased immediately after acid exposure (time 0), with the lowest levels found at 15 to 30 min; levels returned to baseline by 2 h (Fig. 4). Levels of phospho-Cdk2 (Thr^{147/Tyr^{157}}) increased immediately after acid exposure and remained elevated at 2 h; levels returned to baseline by 4 h after exposure (Fig. 4). Cdk2–cyclin E activity (measured as phosphorylation of histone H1) decreased immediately after acid exposure (time 0) with the lowest levels found at 15 to 30 min (Fig. 4). However, there was no difference in Cdk4/6-cyclin D1 activity (measured as phospho-Rb) following acid exposure at any of the
time points examined (Fig. 4). These data suggest that acid causes a rapid decrease in Cdc25A levels, which is accompanied by persistent phosphorylation of Cdk2 and decreased activity of the Cdk2–cyclin E complex. These changes may contribute to the early acid-induced delay in progression through the G1-S checkpoint in BAR-T cells.

Acid exposure increases levels of phospho-Chk2. Chk1 and Chk2 are serine/threonine kinases that, when activated by phosphorylation, transfer phosphate groups to Cdc25A, thereby targeting the protein for degradation (25). Having found a decrease in Cdc25A expression levels, we investigated whether acid exposure activated the Chk pathways. Compared with non–acid-exposed controls, acid exposure resulted in no change in levels of total Chk1, phospho–Chk1-s317 and phospho–Chk1-s345 at any time point (Fig. 5). We also found that levels of total Chk2 did not seem to differ from those of nonexposed controls at any time following acid exposure (Fig. 5). In contrast, phospho-Chk2 levels increased substantially after acid exposure, with maximal expression detected at 15 to 30 min (Fig. 5). These data show that acid activates the Chk2 checkpoint kinase, suggesting that the Chk2 pathway may be involved in the early acid-induced delay in progression through the G1-S checkpoint in BAR-T cells.

Inhibition of the Chk2 pathway prevents the early delay in cell cycle progression following acid exposure. We used caffeine, an inhibitor of the ATM/ATR kinases immediately upstream of Chk2, to determine whether the delay in cell cycle progression induced by acid exposure is dependent on this checkpoint kinase cascade. In preliminary dose response experiments, we determined that 10 mmol/L caffeine prevented acid-induced phosphorylation of Chk2 kinase (Fig. 6A). BAR-T cells were released into growth media for 14 h and then exposed to acid for 10 min in the presence and absence of 10 mmol/L caffeine; flow cytometry was used to analyze movement into the S phase at 2 and 4 h after acid exposure. In the absence of caffeine, acid significantly decreased the fraction of cells in the S phase compared with non–acid-exposed controls (Fig. 6C, top). In caffeine-treated cells, there was no difference in the fraction of cells in the S phase between acid-exposed and nonexposed cells at 2 h (Fig. 6C, top). Treatment with caffeine did not prevent the acid-induced decrease in the fraction of cells in the S phase at 4 h, however (Fig. 6C, top).

We next confirmed our findings by transfecting the BAR-T cells with Chk2 siRNA oligonucleotides. To determine the efficiency of Chk2 siRNA for inhibiting Chk2 expression, Western blotting was done. As expected, we found a marked decrease in Chk2 expression following transfection with the siRNA compared with transfection with a control siRNA (Fig. 6B). We found that Chk2 siRNA prevented the acid-induced decrease in the fraction of cells in S phase at 2 h compared with acid-exposed cells transfected with the control siRNA (Fig. 6C, bottom). Transfection with Chk2 siRNA did not prevent the acid-induced decrease in the fraction of cells in S phase at 4 h, however (Fig. 6C, bottom). These data suggest that Chk2 activation mediates the early (2-h) acid-induced delay in movement from G1 into S phase of the cell cycle, whereas the later (4-h) delay in progression through this checkpoint is independent of Chk2.

Discussion

In contrast to esophageal adenocarcinoma cells in which acid exerts pro-proliferative effects, in telomerase-immortalized, non-neoplastic BAR-T cells, we have found that acid causes a decrease in cellular proliferation that is associated with the activation of the p53 and Chk2 pathways and with alterations in the levels and activities of proteins that regulate the G1-S cell cycle checkpoint. We have also shown that the early, antiproliferative effects of acid are mediated by the activation of the Chk2 pathway. Thus, benign BAR-T cells, with their intact cell cycle checkpoint machinery, respond quite differently than Barrett's cancer cells, which proliferate in response to acid exposure (7, 26, 27).

In an earlier study, we found that BAR-T cells that were subjected to multiple 3-min acid exposures exhibited a significant decrease in cell numbers at 24 h (9). In the present study, we found a similar

Figure 2. A, a representative experiment showing the results of optical morphology for acid-exposed and non–acid-exposed cells; cells irradiated with UV-B at 200 J/m^2 served as a positive control. Note that UV-B irradiation at 200 J/m^2 induced features of apoptosis in the Barrett's cells. No such morphologic features were seen following acid exposure. B, results of acid exposures on apoptosis as determined by annexin V staining. Columns, means of at least five individual experiments; bars, SE. *, P < 0.05 compared with controls.
significant decrease in cell numbers for BAR-T cells after a single, 3- or 10-min exposure to acid. To determine whether this acid-induced decrease in cell number was unique only to the BAR-T cell line, we used two additional telomerase-immortalized, metaplastic Barrett’s cells lines that were established in our laboratory. A similar decrease in cell number was observed in all three lines, suggesting that the antiproliferative effect of acid is a common property of non-neoplastic BAR-T cells.

We next explored whether the acid-induced decrease in cell number was the result of a direct toxic effect of the acid, an acid-triggered increase in apoptosis, or an acid-induced decrease in cell proliferation. We found that acid exposure had no effect on cell viability, and we observed no morphologic features of apoptosis. Using the annexin V assay, however, we found a small but statistically significant increase in the fraction of apoptotic cells following a 10-min acid exposure. We have previously shown that acid exposure times of up to 3 min do not induce apoptosis in BAR-T cells (9). These findings are in contrast to those in esophageal adenocarcinoma cells in which acid exposure was found to induce antiproliferative effects (7, 26). Furthermore, our results suggest that the acid-induced decrease in BAR-T cell numbers was not the result of either direct acid toxicity or apoptosis. We did find that acid prolonged the transition from the G1 into the S phase of the cell cycle, an effect that could be detected as early as 2 h after the acid exposure. The G1-S cell cycle checkpoint is known to be highly susceptible to influence by exogenous factors in the cellular environment (10).

The tumor suppressor p53 and its downstream effector p21 are proteins well known to regulate the transition from G1 to S. In response to genotoxic stress, p53 protein becomes post-translationally modified, stabilized, and able to induce the transcription of genes that can cause cell cycle delay, arrest, and apoptosis (11). In earlier studies, we showed that BAR-T cells express functional p53 and undergo apoptosis in response to UV-B irradiation (9, 13). In the present study, we determined protein expression levels of total p53, phospho-p53 Ser15 (the transcriptionally active form), and phospho-p53 Ser46 (the apoptotically active form) in BAR-T cells following acid exposure. We found an increase in total p53 and phospho-p53 Ser15, but not phospho-p53 Ser46 over a 6-h time course. In accordance with the observed increase in the transcriptionally active form of p53, we also observed an increase in p21 expression. In human glioblastoma cells, acidic conditions have been shown to increase transcriptional activity of p53 that is accompanied by an increase in p21 miRNA and protein expression (28). Therefore, it is likely that the induction of p21 that we observed in our BAR-T cells was due to enhanced transcriptional activity of p53 induced by the acid exposure.

Cyclin D1–Cdk4 and cyclin E–Cdk2, the main cyclin-Cdk complexes that regulate the G1-S checkpoint, are known to be targets for inhibition by p21 (29). We found no apparent effect of acid on cyclin D1–Cdk4 activity. In contrast, acid exposure caused an immediate decrease in cyclin E–Cdk2 activity that reached its lowest levels within 15 to 30 min. This decrease in cyclin E–Cdk2 activity occurred well before we detected any increase in p21 protein levels, suggesting that p21 was not mediating the early acid-induced decrease in cyclin E–Cdk2 activity.

The p53-dependent arrest of cells at the G1-S checkpoint has been called a delayed response because it is a multistep process (involving protein modification, stabilization, accumulation, and transcriptional activation of downstream effector proteins) that can require several hours to exert its effects (10). In contrast, a rapidly activated, p53-independent G1-S checkpoint pathway involving the degradation of Cdc25A recently has been described (17). Normally, Cdc25A removes an inhibitory phosphate group from the Cdk2 kinase, thereby activating the cyclin E–Cdk2 complex and promoting cell cycle progression (11). We found that acid exposure induced a rapid decline in Cdc25A expression levels that corresponded with increased levels of Cdk2 phosphorylated at its inhibitory sites (Thr14/Tyr15).

Cdc25A is targeted for proteasome-mediated degradation when it is phosphorylated by Chk1 or Chk2 (10, 25). We determined whether acid activated Chk1 and Chk2 in BAR-T cells by performing Western blots for expression of the phosphorylated, active forms of these kinases. We found that acid exposure increased phosphorylation of Chk2, but not Chk1. To determine whether the acid-induced delay in G1-S phase progression was Chk2 dependent, we inhibited Chk2 using either a pharmacologic inhibitor, caffeine, or a Chk2 siRNA construct and confirmed inhibition by Western blotting. In contrast to control cells, in which acid exposure caused a decrease in the fraction of cells in the S phase at 2 h, Chk2-inhibited cells exhibited no such decrease at 2 h after acid exposure. However, Chk2 inhibition did not prevent the acid-induced decrease in the fraction of cells in the S phase at 4 h. These observations suggest that the early antiproliferative...
The effect of acid exposure in BAR-T cells is mediated by the activation of Chk2, which results in a rapid loss of Cdc25A activity with failure to activate Cdk2.

We have found that acid activates both the p53 and Chk2 pathways in BAR-T cells. Those pathways are well known to be activated by DNA damage induced by agents such as UV light or ionizing radiation (25). Our findings that acid exposure can trigger those same pathways suggest that acid may be exerting direct genotoxic effects. Acid has been shown to cause DNA damage in Chinese hamster cells and in MDA-MB-231 breast cancer cells (30–32). Biopsies of metaplastic Barrett’s epithelium exhibit high levels of DNA strand breaks and oxidative injury, and cultures of esophageal adenocarcinoma cells exposed to acid and bile acids develop DNA injuries (33–37). We speculate that acid exposure may also cause genotoxic damage in our BAR-T cells. Unlike cancer cells that proliferate in response to acid, however, benign Barrett’s cells have intact cell cycle checkpoint mechanisms (Chk2 and p53) that are activated by acid-induced DNA damage to decrease cell proliferation.

Our finding that acid exerts antiproliferative effects on BAR-T cells has interesting clinical implications. In an effort to prevent cancer for patients with Barrett’s esophagus, clinicians commonly prescribe antisecretory medications in doses beyond those required to heal the symptoms and endoscopic signs of reflux esophagitis. This practice is based on the *in vitro* studies discussed above and on clinical studies showing decreased proliferation markers in esophageal biopsy specimens from patients with Barrett’s esophagus who received aggressive antisecretory therapy.
In those clinical studies, it is not possible to determine whether acid control decreased proliferation in Barrett's epithelium directly by removing an acid-triggered proliferation signal, or indirectly, through the anti-inflammatory effects of healing reflux esophagitis. Inflammation is well known to be a potent stimulus for proliferation and carcinogenesis in a number of organs (39). In our BAR-T cell cultures, we have found that acid exerts direct, antiproliferative effects via the activation of the p53 and Chk2 pathways. In clinical studies, those potentially beneficial anti-proliferative effects of acid may have been overshadowed by the more potent antiproliferative effects of acid suppression in healing the inflammation of reflux esophagitis.

Further studies elucidating the mechanisms by which acid triggers the p53 and Chk2 pathways in BAR-T cells will be important to help determine what is the optimal level of acid suppression for patients with Barrett's esophagus. If those pathways are activated because acid causes DNA damage, then aggressive acid-suppressive therapy, even to the point of complete elimination of acid secretion, may be appropriate. If, however, acid directly triggers antiproliferative pathways without causing DNA damage, then it may be prudent to tailor anti-secretory therapy to achieve a level of gastric acid suppression that heals esophagitis without completely eliminating acid secretion.

In conclusion, we have shown that acid exposure decreases cell proliferation by delaying progression through the G1-S cell cycle checkpoint in non-neoplastic BAR-T cells. Acid exposure rapidly decreases the expression of phospho-Chk2, decreases the levels and activity of Cdc25A, and decreases cyclin E–Cdk2 activity. Hours later, acid exposure results in increased levels and transcriptional activation of p53 along with increased expression of p21. Moreover, activation of Chk2 mediates the early but not the late acid-induced delay in G1-S cell cycle progression in metastatic Barrett's cells. These studies have elucidated mechanisms whereby acid can exert disparate effects on proliferation in neoplastic and non-neoplastic BAR-T cells.

Acknowledgments

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