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p53/HMGB1 Complexes Regulate Autophagy and Apoptosis

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Abstract

The balance between apoptosis (“programmed cell death”) and autophagy (“programmed cell survival”) is important in tumor development and response to therapy. Here we show that HMGB1 and p53 form a complex which regulates the balance between tumor cell death and survival. We demonstrate that knockout of p53 in HCT116 cells increases expression of cytosolic HMGB1 and induces autophagy. Conversely, knockout of HMGB1 in mouse embryonic fibroblasts increases p53 cytosolic localization and decreases autophagy. p53 is thus a negative regulator of the HMGB1/Beclin 1 complex, and HMGB1 promotes autophagy in the setting of diminished p53. HMGB1-mediated autophagy promotes tumor cell survival in the setting of p53-dependent processes. The HMGB1/p53 complex affects the cytoplasmic localization of the reciprocal binding partner thereby regulating subsequent levels of autophagy and apoptosis. These insights provide a novel link between HMGB1 and p53 in the crossregulation of apoptosis and autophagy in the setting of cell stress, providing insights into their reciprocal roles in carcinogenesis.

Keywords

HMGB1; p53; Autophagy; Apoptosis; Colorectal cancer

INTRODUCTION

Autophagy maintains cellular viability in the setting of various stressors (1, 2). Mediated via the lysosomal degradation pathway, autophagy recycles cellular proteins and organelles to ensure cell survival. Apoptosis is a process through which superfluous, effete, ectopic, aged, damaged, unattached, or mutated cells are eliminated (3). The balance between autophagy and apoptosis is regulated by various cell signals, and crosstalk between these pathways

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determines cell fate in the setting of stress (4, 5). In addition to their role in respectively suppressing or promoting tumorigenesis, apoptosis and autophagy contribute to the sensitivity or resistance of tumors to various therapies (6, 7). A more detailed understanding of the mechanisms by which autophagy interfaces with apoptotic mechanisms will enhance our understanding of cancer biology (8, 9).

p53 and associated molecular pathways are the most commonly mutated in human cancers, regulating apoptosis, autophagy, metabolism, and persistence in hypoxic environments (10). p53 itself promotes apoptosis by both transcription-dependent and independent mechanisms (11-13). In contrast, p53 regulates autophagy in a dual fashion whereby the pool of cytoplasmic p53 protein represses autophagy in a transcription-independent fashion (14), and the pool of nuclear p53 stimulates autophagy in a transcription-dependent fashion (15, 16). These studies suggest that the function of p53 in the regulation of autophagy (and cell fate) is tightly controlled by its subcellular localization (17).

High mobility group box 1 (HMGB1), a highly conserved nuclear protein, acts as a chromatin-binding factor that bends DNA and promotes access to transcriptional protein complexes. In addition to its nuclear role, HMGB1 also functions as an extracellular signaling molecule during inflammation, cell differentiation, cell migration, wound healing, and tumor progression (18, 19). Our recent studies demonstrate that endogenous and exogenous HMGB1 are critical regulators of autophagy (20-24). Endogenous HMGB1 promotes autophagy by both transcription-dependent and independent mechanisms (22, 23). Cytoplasmic HMGB1 is a Beclin 1-binding protein, which sustains Beclin 1-PtdIns3KC3 complex activation during upregulation of autophagy (23, 25). Additionally, exogenous HMGB1 promotes autophagy in tumor cells through interactions with the receptor for advanced glycation end products (RAGE) (26, 27).

The relationship between HMGB1 and p53 in the regulation of autophagy was previously unknown. Here, we demonstrate that HMGB1 and p53 form a complex that regulates the cytoplasmic localization of the binding protein and subsequent levels of autophagy. p53^{-/-} HCT116 cells rapidly increase cytosolic HMGB1 and associated autophagy in response to stress. Conversely, HMGB1^{-/-} mouse embryonic fibroblasts (MEFs) have increased cytosolic translocation of p53, enhanced apoptosis, and decreased autophagy in response to stress. Thus, HMGB1 reciprocally complements and modulates the functions of p53 in response to cellular stress.

MATERIALS AND METHODS

Reagents

Antibodies to p53 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technology (Danvers, MA, USA) and DakoCytomation (Denmark). The antibodies to p62 were from Novus Biologicals (Littleton, CO, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies to microtubule associated protein light chain 3 (LC3)-I/II and DRAM were from Novus. The antibody to tubulin was from Sigma (St. Louis, MO, USA). The antibodies to actin, Bax, COX IV, ULK1, CHOP and calnexin were from Cell Signaling Technology. The antibodies to fibrillarin and PCNA were from Abcam (Cambridge, MA, USA). The mouse monoclonal antibodies to HMGB1 were from Novus Biologicals (Littleton, CO, USA) and the affinity-purified polyclonal rabbit antibodies to HMGB1 that recognizes a specific HMGB1 peptide (166-172) was generated for our laboratory on contract (Sigma Antibody Service, St. Louis, MO). The RAGE antibodies were from Medimmune (Gaithersburg, MD, USA).

The purified mouse IgG2a, κ isotype control was from BD Biosciences (San Jose, CA, USA). The purified rabbit IgG was from Jackson ImmunoResearch Laboratories, INC (West Grove, PA, USA). The recombinant Mdm2 protein was from Novus Biologicals. The recombinant p53 protein was from Sigma. Recombinant p53 and GnRH-p53 were obtained from GeneCopoeia, Inc (Rockville, MD, USA). Recombinant HMGB1 proteins, noted Lilly Pool 2 (LP2) and Lilly Pool 3 (LP3) were obtained from Eli Lilly Company (Robert Bentschop, Indianapolis, Indiana, USA) as previously described (20). Purified HMGB1 was a kind gift of Richard Shapiro and Timothy Billiar (University of Pittsburgh). The recombinant HMGB1 A box and B box were kind gifts from Kevin J. Tracey (North Shore University Hospital). Purified RAGE was a kind gift from Tim D.Oury (University of Pittsburgh). All other reagents were obtained from Sigma.

Cell culture

Wildtype and p53^{-/-} HCT116 human colorectal cancer cell lines were a kind gift of Bert Vogelstein (Johns Hopkins, Baltimore, MD). p53 mutant DLD1 (protein: S241F; coding sequence: 722C>T) and HT29 (protein: R273H, coding sequence: 818G>A) human colorectal cancer cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). Wildtype and HMGB1^{-/-} immortalized mouse embryonic fibroblasts were obtained from Marco E. Bianchi (San Raffaele Institute, Milan, Italy). All cell lines were cultured in McCoy's 5A or IMDM medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified incubator with 5% CO₂ and 95% air. All cell lines were characterized and confirmed according to ATCC instructions.

Biolayer interferometry

All measurements were made using a FortéBio Octet QK platform and default settings for the sample stage orbital rate (1000 rpm) and temperature (30 °C) were used.

Streptavidin Biosensors: Prior to assay, p53 or HMGB1 was biotinylated using a 5:1 biotin:protein ratio using the EZ-Link LC-LC (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Unincorporated biotins were removed using Zeba desalt columns (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Biosensors were equilibrated in PBS, loaded with various nM concentrations of biotinylated protein in PBS, equilibrated in kinetics buffer (Menlo Park, CA), then introduced into solutions containing various nM concentrations of protein in kinetics buffer or buffer alone and washed with kinetics buffer. The association constant, K_a , and dissociation constant, K_d , were calculated using the FortéBio software (Menlo Park, CA, USA), controlling for the buffer only sample.

Amine Reactive Biosensors: Amine reactive biosensors were equilibrated in a 100mM MES solution. Biosensors were activated with a 1:50 0.4M EDC/NHS:MES solution. The ligand of interest was then introduced at various concentrations. 1 μ M Glycine was then used for quenching and PBS used to equilibrate the protein. The K_a was calculated upon introduction of the second ligand. The K_d was calculated on introduction of the tip into PBS using the FortéBio software as noted above.

Survival clonogenic assay

Long-term cell survival was monitored in a colony formation assay. In brief, 1000 cells were treated with individual chemotherapeutic drugs for 24 h and plated into 24 well plates. Colonies were visualized by crystal violet staining after 2 weeks as previously described (28).

Full Methods are available in the SUPPLEMENT MATERIALS AND METHODS.

RESULTS

HMGB1 binds p53 within the nucleus and cytosol

Although interactions between HMGB1 and p53 have been previously well defined in the nucleus, these *in vitro* assays were only able to demonstrate an interaction in the presence of DNA (29). We evaluated the direct interaction between HMGB1 and p53 using biolayer interferometry (30). We coupled recombinant HMGB1 to an amine reactive biosensor using an amine linking reagent, introduced recombinant p53 to determine the association constant, and then washed off the p53 to determine the dissociation constant. We found in this cell free assay that in the absence of DNA, the K_D for HMGB1 and p53 binding was $1.15 \times 10^{-9} \pm 0.03$ M (Supplemental Table 1). Similarly, when p53 was coupled to the biosensor and recombinant HMGB1 was introduced in solution, the K_D was determined to be $1.83 \times 10^{-9} \pm 0.44$ M (Supplemental Table 1). Furthermore, oxidation of HMGB1 with H_2O_2 had a minimal effect on the affinity with p53 whereas reduction of p53 using tris 2-carboxyethyl phosphine (TCEP) abrogated interaction with HMGB1 (Supplemental Table 2). Others have demonstrated that the A box of HMGB1 interacts with p53 (31). Thus, we coupled the A or B box of HMGB1 to the biosensor and then determined the affinity for p53. We found that the A box had a slightly higher affinity for p53 than the B box with calculated K_D 's of 6.38×10^{-9} M and 14.5×10^{-9} M respectively (Supplemental Table 3).

To validate these findings using the amine reactive biosensors in the analysis of p53/HMGB1 interactions, we performed the assay with known targets and nonspecific targets for these respective proteins. HMGB1 exhibited a K_D of 3.3×10^{-8} M for soluble RAGE, a receptor for HMGB1, but did not bind to IL-2 or bovine serum albumin (BSA, Supplemental Table 4). Similarly, p53 exhibited a K_D of 1.87×10^{-9} M for murine double minute-2 (Mdm2) which binds and ubiquitinates p53, but did not bind to BSA (Supplemental Table 4). To further confirm the interaction between p53 and HMGB1 using biolayer interferometry we biotinylated HMGB1 and p53, coupled the biotinylated protein to streptavidin biosensors and then determined the affinity for the target protein. The association and dissociation curves for biotinylated p53 and a dilution series of HMGB1 (Figure 1A) and biotinylated HMGB1 and a dilution series of p53 (Figure 1B) were used to determine the global fit for the equilibrium dissociation constants. Biotinylated HMGB1 demonstrated a K of 9.47×10^{-9} D ± 1.47 M for p53 and biotinylated p53 demonstrated a K of 7.35×10^{-8} D ± 8.10 M for HMGB1 (Supplemental Table 5).

To determine the dynamic interaction between HMGB1 and p53 *in vitro* in response to cell stress, we starved HCT116 cells to enhance levels of autophagy. We then immunoprecipitated whole cell lysates with HMGB1 antibody and probed for p53 by western blot. We found increased complex formation between HMGB1 and p53 following Hank's balanced salt solution (HBSS)-induced starvation by immunoprecipitation assay (Figure 1C). Moreover, immunoprecipitation of nuclear and cytosolic extracts revealed HMGB1 and p53 binding in both subcellular compartments, especially in the nucleus following HBSS-induced starvation (Figure 1D). Furthermore, confocal microscopy revealed significant colocalization of HMGB1 with p53 within the nucleus and cytosol following HBSS-induced starvation (Figure 1E).

Loss of p53 enhances autophagy and promotes cytosolic HMGB1 translocation

Others have shown that knockout of p53 increases starvation induced autophagy (14). We confirmed this finding in p53^{-/-} HCT116 cells by western blot analysis of p62/ sequestome 1 and microtubule associated light chain 3 (LC3) to monitor levels of autophagy. When autophagy is upregulated, LC3 is cleaved (LC3-I) and then conjugated to phosphatidylethanolamine (LC3-II), which is recruited to the autophagosome. p62 is a

scaffolding protein that delivers ubiquitinated proteins to the autophagosome and is itself degraded following fusion with the lysosome. HCT116 p53^{-/-} cells had increased autophagy, as demonstrated by increased levels of LC3-II (Figure 2A), accumulation of LC3 punctae (Figure 2B), and decreased levels of p62 (Figure 2A) under basal conditions relative to p53^{+/+} cells. When p53^{-/-} cells were starved to induce autophagy, there was a further increase in autophagy (Figure 2A-B). Notably, p53^{-/-} cells had increased levels of HMGB1 in the cytosol (“cyt-HMGB1”) under basal conditions and in response to starvation (Figure 2C). Moreover, in the DLD-1 and HT-29 colorectal cancer cell lines with endogenous mutant p53 (32) there was increased HMGB1 cytoplasmic translocation (Figure 2C). Our previous study demonstrated that cyt-HMGB1 interacts with Beclin 1, an important autophagy-related protein in human cells (25), thereby promoting autophagy (23). As expected, increased complex formation between HMGB1 and Beclin 1 was detected in p53^{-/-} cells (Figure 2D). To explore the role of HMGB1 in p53-associated autophagy, we suppressed HMGB1 expression by specific shRNA in p53^{-/-} cells. Knockdown of HMGB1 attenuated p53 deficiency-induced autophagy as detected by analysis of LC3 punctae using confocal microscopy (Figure 2E) and ultrastructural analysis of autophagosome-like structures using transmission electron microscope (Figure 2F). Pifithrin- α (“PFT- α ”), a pharmacological antagonist of p53, induces autophagy in HCT116 cells (33), and promoted HMGB1 cytosolic translocation (Figure 2G). The HMGB1 inhibitor, ethyl pyruvate (“EP”) (34), diminished PFT- α induced cytosolic translocation of HMGB1 and autophagy (Figure 2G). These results suggest that p53 inhibits autophagy by regulating the subcellular localization of HMGB1. In the absence or inhibition of p53, increased HMGB1 cytosolic translocation results in increased levels of autophagy.

Loss of HMGB1 leads to increased p53 cytosolic translocation and decreased autophagy

Starvation of HCT116 HMGB1 knockdown cells decreased levels of autophagy as demonstrated by decreased LC3-II expression (Figure 3A) and LC3 punctae (Figure 3B) and increased p62 expression (Figure 3A). To determine if the reduced levels of autophagy correlated with changes in p53 localization, we performed a western blot analysis of p53 in nuclear and cytosolic fractions of HMGB1^{+/+} and HMGB1^{-/-} MEFs. Indeed, HMGB1^{-/-} cells had increased levels of p53 in the cytosol and decreased levels of p53 in the nucleus relative to HMGB1^{+/+} cells (Figure 3C). Furthermore, when HMGB1^{-/-} cells were starved to induce autophagy, there was a further increase in cytosolic p53 and decrease in nuclear p53 (Figure 3C). These findings suggest that HMGB1 and p53 interactions in the nucleus serve to limit the level of cytosolic p53. In the absence of HMGB1, there is increased p53 translocation to the cytosol which itself appears to limit levels of autophagy. To explore whether p53 is required for HMGB1-mediated autophagy, we knocked down p53 in HMGB1^{+/+} and HMGB1^{-/-} cells. Interestingly, knockdown of p53 did not restore LC3 punctae formation in HMGB1^{-/-} cells (Figure 3D), suggesting that p53 is not required for HMGB1-sustained autophagy.

p53-mediated expression of DRAM and ULK1 is HMGB1-independent

Damage-regulated autophagy modulator (DRAM) is a p53 target gene encoding a lysosomal protein that promotes autophagy (15). Unc-51-like kinase 1 (ULK1) is important for induction of autophagy and is a direct target of p53 (16). To explore whether the interaction of HMGB1/p53 within the nucleus influences p53-dependent expression of DRAM and ULK1, we performed a western blot analysis of p53^{-/-} and HMGB1 knockdown HCT116 cells. Consistent with previous studies (15, 16), DRAM and ULK1 were induced by DNA damaging agents such as adriamycin (“ADM”) and etoposide (“ETO”) (Figure 4A-B). Knockout of p53, but not knockdown of HMGB1, impaired upregulation of DRAM and ULK1 (Figure 4A-B), suggesting that p53-mediated expression of DRAM and ULK1 during DNA damage is HMGB1-independent.

HMGB1-mediated autophagy promotes cell survival during p53-dependent apoptosis

There is a tight and complex relationship between apoptosis and autophagy (4), with both processes increasing during periods of cellular stress. Autophagy plays a dual role in the regulation of p53-dependent apoptosis, as it is reported to both promote and inhibit cellular death (15, 16,35) depending on cell type, the tissue microenvironment, and response to other stimuli. To explore the role of HMGB1 in p53-dependent apoptosis, we suppressed HMGB1 expression by shRNA in HCT116 cells (Figure 2E). Knockdown of HMGB1 restored and increased the sensitivity of p53^{-/-} and p53^{+/+} cells respectively to ADM and ETO-induced apoptosis as evaluated by flow cytometry (Figure 5A) and in a survival clonogenic assay (Figure 5B and 5C). Moreover, knockdown of HMGB1 increased Bax translocation from the cytosol to mitochondria, cytochrome c release from mitochondria, and caspase-9 activation in p53^{-/-} cells (Figure 5D-E), which are downstream events in the p53 apoptosis pathway (36). In contrast, knockdown of HMGB1 decreased autophagy in p53^{-/-} and p53^{+/+} cells (Figure 5F). Furthermore, the autophagy inhibitors 3-methyladenine (“3-MA”) and wortmannin (“Wort”) increased ADM and ETO induced apoptosis in p53^{-/-} cells (Figure 5G). These findings suggest that HMGB1-mediated autophagy promotes cell survival during p53-dependent apoptosis.

HMGB1 regulates p62 degradation during autophagy—p62 is a multifunctional protein that regulates cell proliferation, differentiation, apoptosis, inflammation, autophagy and obesity (37, 38). p62 binds protein aggregates and is degraded by autophagy (39). In contrast, p62 upregulation is observed with increased endoplasmic reticulum (ER) stress (40, 41), although ER stress triggers LC3 conversion and autophagy (41, 42). ER stress and upregulation of p62 are common in human tumors (38, 43,44). To explore whether HMGB1 regulates ER stress-mediated p62 expression, we treated cells with thapsigargin which induces ER stress (Figure S1D). Knockdown of HMGB1 by shRNA inhibited thapsigargin induced upregulation of ER stress markers (e.g., calnexin and CHOP), LC3-II and p62 expression. In contrast, knockdown of HMGB1 limited starvation-induced p62 degradation (Figure 3A). These findings suggest that HMGB1 not only regulates p62 expression in response to ER stress, but also p62 degradation during autophagy.

Subcellular localization of p53 and HMGB1 in human colon cancer

To assess the clinical significance of complex formation between HMGB1 and p53, and the subsequent regulation of the subcellular localization of these proteins in the regulation of autophagy, we analyzed a tissue microarray from patients with normal colon, normal adjacent colonic tissue to tumors, colonic adenomas, and invasive adenocarcinomas. Normal and normal adjacent tissues demonstrated nuclear localization of HMGB1 and undetectable levels of p53 (Figure S1A, **top left and right panels**). Tissue from colon adenomas, however, demonstrated increased expression of HMGB1 in the cytosol and within the nucleus (Figure S1A, **bottom, left panel**). Furthermore, tissue from patients with colon cancer had an even further increase in HMGB1 in the cytosol, accompanying increased p53 expression (Figure S1A, **bottom, right panel**). There was significantly greater overall total HMGB1 (n=8, paired T-test, p =0.00031) and nuclear HMGB1 (n=8, paired T-test, p =0.023) found in the samples from patients with adenocarcinoma when compared with normal tissues (Figure S1C).p62 levels correlated with changes in HMGB1 expression as the normal colon and normal adjacent tissues demonstrated lower expression of cytosolic p62 (Figure S1B **top left and right panels**). The colon adenoma and adenocarcinoma tissues (Figure S1B **bottom left and right panels**) demonstrated increased cytosolic p62 and overall RAGE expression relative to the normal and normal adjacent tissues (Figure S1B **top left and right panels**). Adenocarcinomas had significantly greater p62 expression than adenomas and normal adjacent tissues from matched patients (n= 8,ANOVA, p = 0.00026). Adenocarcinomas also had significantly greater RAGE expression than adenomas and

normal adjacent tissues from matched patients (n=8, ANOVA, $p = 0.00652$). Linear regression was used to determine if HMGB1 or p53 expression was associated with survival (Supplemental Table 6). p53 expression had a statistically significant association with time of survival after first recurrence ($p < 0.00761$) by automated and manual scoring. Nuclear p53 expression demonstrated a positive trend with survival time from diagnosis that was not statistically significant ($p = 0.059$, manual scoring). Others have shown that HMGB1 expression correlates with tumor progression and negatively impacts survival (37). We demonstrated that nuclear HMGB1 expression had a positive trend with survival time from diagnosis ($p = 0.068$, automated scoring). These results suggest that the subcellular localization of HMGB1 and p53 are likely related to their roles in regulating survival. *In vitro* cultures of HMGB1 knockdowns of the colorectal cancer, HCT116 are also consistent with a role for HMGB1 in regulating autophagy (Figure S1D).

DISCUSSION

p53 expression is linked to regulation of both autophagy and apoptosis. The mechanism by which it either promotes or restricts these important cellular processes remains controversial. In this study, we demonstrate that HMGB1/p53 complexes regulate the cytoplasmic localization of the reciprocal protein and subsequent levels of autophagy and apoptosis (Figure 6). We have demonstrated that redox chemistry is critical to the interaction between p53 and HMGB1, such that reduction of p53 abrogates binding. HMGB1 sustains autophagy in the setting of oxidative stress (45). Reactive oxygen species induce HMGB1 cytoplasmic translocation to promote and sustain autophagic flux (21). Therefore, increased production of reactive oxygen species following stress may enhance p53 and HMGB1 binding and thus provide a fine balance that regulates levels of autophagy and apoptosis.

We observed increased cytosolic HMGB1 in p53^{-/-} cells and increased cytosolic p53 in HMGB1^{-/-} cells relative to wild type cells. This suggests that complexes between HMGB1 and p53 sequester the binding partner within the nucleus and that without a binding partner, HMGB1 or p53 can more readily translocate to the cytosol. As a transcription factor, p53 activates genes that induce apoptosis, permanent cell cycle arrest (senescence) or autophagy (e.g. DRAM and ULK1) (15, 16). As a cytoplasmic protein, p53 mediates tonic inhibition of autophagy (14) and the induction of apoptosis, the latter presumably through coupling with the PUMA/Bax-mediated mitochondrial pathway (11, 13). In contrast, both nuclear and cytoplasmic HMGB1 are positive regulators of autophagy. Cytoplasmic HMGB1 interacts with Beclin 1, and localizes Beclin 1 to autophagosomes (23). We demonstrated that loss of p53 increases interactions between HMGB1 and Beclin 1, suggesting that p53 is a negative regulator of HMGB1/Beclin 1 interactions. On the other hand, as a transcription factor, HMGB1 regulates the expression of the small heat shock protein 27 (Hsp27 or HSPB1), which we have shown regulates the cytoskeleton as well as the dynamics of autophagy and mitophagy (22). HMGB1 does not, however, influence p53-dependent expression of DRAM and ULK1 during autophagy. In contrast, HMGB1 is required for induction of autophagy during knockout or pharmacological inhibition of p53. These studies suggest that HMGB1 regulates the cytoplasmic but not the nuclear functions of p53 during autophagy.

Additionally, we demonstrate that HMGB1-mediated autophagy contributes to apoptosis resistance in p53^{-/-} cancer cells. As a tumor suppressor, p53 limits tumor cell growth by inducing cell cycle arrest and apoptosis in response to cellular stress such as DNA damage and oncogene activation. The inhibition of autophagy is an attractive therapeutic option, as there is increasing evidence suggesting that inhibiting autophagy in cancer cells is associated with increased apoptotic cell death (9). Others have suggested, that in the absence of apoptosis, autophagic cell death can be an alternative form of cell death by excessive self-digestion (46). However, increasing studies indicate that the accumulation of

autophagosomes in dying cells is not the important effector mechanism of cell death (47). Indeed, autophagy is a response to stress associated with cell death whereby it functions primarily as a cell survival mechanism (1). In this sense, the term “autophagic cell death” may be inappropriate (47), whereas autophagy occurring prior to various forms of cell death (apoptosis, necroptosis, necrosis) may be a better way to consider how these events relate to one other. We demonstrated that HMGB1 is required for autophagy in p53^{-/-} cells, and that knockdown of HMGB1 decreases autophagy and increases apoptosis in p53^{-/-} cells. Thus, HMGB1 is an important regulator of p53 functions and represents a novel therapeutic target for drug resistance in p53 deficient cells.

HMGB1/p53 interactions are important in the transition from normal tissue to adenocarcinoma in colon cancer. HMGB1 is localized to the nucleus in healthy and normal tissue adjacent to tumors but increased within the cytosol of colonic adenomas and colon cancer tissues. Others have shown significantly higher HMGB1 mRNA expression in Dukes' C colorectal cancer when compared with normal tissues indicating that there is both increased mRNA and protein expression of HMGB1 with increasing stage of colon cancer (48). Others have also demonstrated that increased HMGB1 correlates with tumor progression and poor prognosis (49). Although we demonstrated a trend relating nuclear HMGB1 expression to patient survival, this did not reach statistical significance. This is most likely due to limited power in our study as there were 119 samples from 29 patients analyzed. Future studies will be important in relating our findings to clinical outcomes. However, our findings suggest that subcellular localization rather than total expression may correlate best with survival. p53 expression remained undetectable in all tissues other than colonic tumors, consistent with its role late in carcinogenesis. Furthermore, p62 expression appeared to correlate with the changes in HMGB1 as it increased in the cytosol in the colon adenoma and carcinoma tissues. The upregulation of p62 is likely directly related to changes in HMGB1 expression as we demonstrated that HMGB1 expression is required to promote ER stress mediated upregulation of p62. These findings indicate that the changes in p53 and HMGB1 expression correlate with increasing ‘stress’ within the tumor microenvironment. Additional work is necessary to explore the nature of HMGB1/p53 interactions in the cytosol, identify other chaperone or binding proteins, and the role of these proteins in regulating mitochondrial function and bioenergetics as well as their connection to immunity (50).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. HMGB1 directly binds p53 (A, B)

StreptavidinBiosensors were loaded with solutions of 25 μ g/mL of biotinylated p53 (A) or HMGB1 (B), and then introduced into solutions containing the nM concentrations of HMGB1 (A) or p53 (B) noted in the bottom panel. Dissociation was assessed by washing biosensors in PBS. The association and dissociation curves are depicted for a representative assay that has been controlled for the sample with kinetics buffer alone. (C, D) HCT116 cells were treated with HBSS for 2h. Cells were lysed in ice-cold RIPA buffer (C), nuclear (“Nuc”) and cytosolic (“Cyt”) isolation (D) was performed using the NE-PER kit (Pierce, USA) and lysates were immunoprecipitated (“IP”) as indicated. The precipitated proteins were subsequently immuno-blotted (“IB”) with the indicated antibodies. Equal loading of samples was confirmed by western blot analysis of each fraction with antibodies specific for a nuclear (Fibrillarin) or cytoplasmic (Tubulin) protein. (E) Colocalization between p53 and HMGB1 in HCT116 cell with or without HBSS treatment for 2h as analyzed by confocal microscopy. All data are representative of two or three experiments.

Figure 2. HMGB1 and p53 Coordinately Regulate Autophagy

(A, B) HCT116 p53^{+/+} and p53^{-/-} cells were treated with or without HBSS for 2h, then p62 degradation and LC3 turnover was analyzed by western blot (A). Bar graphs represent mean protein band intensity ("AU": Arbitrary Units, mean $\pm$ s.d., n = 3; * = $p < 0.05$). In parallel, LC3 punctae were analyzed by confocal microscopy (mean $\pm$ s.d., n = 3; * = $p < 0.05$). (C) HCT116 p53^{+/+}, HCT116 p53^{-/-}, DLD-1, and HT-29 cells were treated with or without HBSS for 2h, then western blot analysis of HMGB1 was performed on cytoplasmic ("Cyt") and nuclear ("Nuc") extracts. (D) Co-IP analysis of the interaction between HMGB1 and Beclin 1 in HCT116 cells treated with or without HBSS for 2h. (E, F) Knockdown of HMGB1 decreases p53 knockout-induced autophagy with or without HBSS treatment for 2h in HCT116 cells evaluated by LC3 punctae using confocal microscopy (mean $\pm$ s.d., n = 3; * = $p < 0.05$) or ultrastructural analysis using TEM. Autophagosome-like structures are denoted with star symbols, "*". (G) HMGB1 inhibitor, ethyl pyruvate (EP, 10 mM), decreases p53 inhibitor, pifithrin- α (PFT- α , 30 μ M), induced autophagy at 24h in HCT116 cells as evaluated by LC3 punctae using confocal microscopy (mean $\pm$ s.d., n = 3; $P < 0.05$).

Figure 3. Stress Induces and Sustains Autophagy in HMGB1 Expressing Cells

(A, B) HCT116 cells transfected with control (ctrl) shRNA and HMGB1 shRNA were treated with or without HBSS for 2h, then p62 degradation and LC3 turnover was analyzed by western blot (A). Bar graphs represent mean protein band intensity ("AU": Arbitrary Units, mean $\pm$ s.d., $n = 3$; $* = p < 0.05$). In parallel, LC3 punctae were analyzed by confocal microscopy (mean $\pm$ s.d., $n = 3$; $* = p < 0.05$). (C) Western blot analysis of indicated proteins in cytoplasmic ("Cyt") or nuclear ("Nuc") lysates from HMGB1^{+/+} and HMGB1^{-/-} MEFs with or without HBSS treatment for 2h. Bar graph represents mean protein band intensity ("AU": Arbitrary Units, mean $\pm$ s.d., $n = 3$; $* = p < 0.05$). (D) p53 was knocked down by siRNA in HMGB1^{+/+} and HMGB1^{-/-} MEFs, then cells were treated with or without HBSS for 2h. Following treatment, LC3 punctae were analyzed by confocal microscopy (mean $\pm$ s.d., $n = 3$; $* = p < 0.05$).

Figure 4. p53 Expression Promotes Apoptosis Following Chemotherapy Treatment

p53 knockout (A) and HMGB1 knockdown (B) HCT116 cells were treated with 0.2 μ g/ml adriamycin (ADM), or 10 μ M etoposide (“ETO”) for 24h. Following treatment, the indicated protein levels were analyzed by western blot. Data are representative of two independent experiments.

Figure 5. Chemotherapy Induced Apoptosis is Limited by HMGB1 Expression

(A-E) HCT116 cells were treated with 0.2 μg/ml adriamycin (ADM), or 10 μM etoposide (“ETO”) for 24h. After treatment, apoptosis was assessed by Annexin V positive staining (A), translocation of Bax and cytochrome C (D) and caspase 9 activity (E) as described in Methods. (B) Cell survival was assessed by a clonogenic assay with a representative image depicted. (C) The dose response curves for ADM and ETO were completed by clonogenic assay (Data is shown as mean ± s.d., n = 3) (F) Levels of autophagy were assessed by LC3 punctae (Data is shown as mean ± s.d., n = 3; * = $p < 0.05$). (G) p53^{-/-} HCT116 cells were treated with 0.2 μg/ml adriamycin (ADM), or 10 μM etoposide (“ETO”) with or without 5mM 3-methyladenine (“3-MA”), 100 nM wortmannin (“Wort”), or both for 24h. After treatment, apoptosis was analyzed by counting Annexin V positive cells by flow cytometry (mean ± s.d., n = 3; * = $p < 0.05$). In parallel, LC3 punctae were analyzed by confocal microscopy (mean ± s.d., n = 3; * = $p < 0.05$).

Figure 6. The Relationship Between HMGB1 and p53 in the Regulation of Autophagy and Apoptosis

Stress signals such as starvation and DNA damage promote interactions between p53 and HMGB1 in the nucleus (“Nuc”) and cytoplasm (“Cyt”). The level of p53/HMGB1 complexes regulates the balance between autophagy and apoptosis in cells. Loss of p53 increases cytosolic HMGB1 and autophagy, and decreases apoptosis. In contrast, loss of HMGB1 increases cytosolic p53 and apoptosis, and decreases autophagy.