Histone H2AX Is a Mediator of Gastrointestinal Stromal Tumor Cell Apoptosis following Treatment with Imatinib Mesylate

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract and are caused by activating mutations of the KIT or platelet-derived growth factor receptor \( \alpha \) (PDGFRA) tyrosine kinases. GISTs can be successfully treated with imatinib mesylate, a selective small-molecule protein kinase inhibitor that was first clinically approved to target the oncogenic BCR-ABL fusion protein kinase in chronic myelogenous leukemia, but which also potently inhibits KIT and PDGFR family members. The mechanistic events by which KIT/PDGFRA kinase inhibition leads to clinical responses in GIST patients are not known in detail. We report here that imatinib triggers GIST cell apoptosis in part through the up-regulation of soluble histone H2AX, a core histone H2A variant. We found that untreated GIST cells down-regulate H2AX in a pathway that involves KIT, phosphoinositide-3-kinase, and the ubiquitin/proteasome machinery, and that the imatinib-mediated H2AX up-regulation correlates with imatinib sensitivity. Depletion of H2AX attenuated the apoptotic response of GIST cells to imatinib. Soluble H2AX was found to sensitize GIST cells to apoptosis by aberrant chromatin aggregation and a transcriptional block. Our results underscore the importance of H2AX as a human tumor suppressor protein, provide mechanistic insights into imatinib-induced tumor cell apoptosis and establish H2AX as a novel target in cancer therapy.

Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract and are caused by activating mutations of the KIT or platelet-derived growth factor receptor \( \alpha \) (PDGFRA) tyrosine kinases (1–3). GISTs can be successfully treated with imatinib mesylate (imatinib, STI-571, Gleevec; Novartis Pharma, Basel, Switzerland; ref. 4), a selective small-molecule protein kinase inhibitor that was first clinically approved to target the oncogenic BCR-ABL fusion protein kinase in chronic myelogenous leukemia (5), but which also potently inhibits the PDGFR family members (PDGFRA, PDGFRB) and KIT (6).

Despite high initial response rates, durable complete responses are rare, and the majority of GIST patients acquire resistance to imatinib over time (7). The mechanistic events by which KIT/PDGFR kinase inhibition leads to clinical responses in GIST patients are not known in detail. By identifying the biochemical mediators of imatinib-induced GIST cell death, it might be possible to develop innovative strategies to induce more complete responses, overcome imatinib resistance, and to enable more effective disease control with an aim toward cure. Moreover, a better understanding of the mode of action of imatinib may help to identify other tumor types that can be treated with small-molecule protein kinase antagonists.

Histone variant H2AX is a major regulator of the cellular response to DNA damage and has previously been shown to be associated with cell death after cytotoxic therapy (8). H2AX differs from core histone H2A by a unique COOH-terminal tail that contains a highly conserved SQE motif with a serine residue at position 139 (9). Ser\(^{139}\) becomes rapidly phosphorylated in response to DNA damage, and protein kinases that have been implicated in this event include ataxia-telangiectasia mutated (ATM), ATM and Rad3-related and DNA-dependent protein kinase (10–12). The phosphorylated form of H2AX has also been referred to as \( \gamma \)-H2AX (13). Histone H2AX is not essential for the initial recognition of DNA damage (14), but it governs the DNA damage response through interactions with proteins that recognize phosphorylated H2AX such as MDC1 (15). H2AX has recently been implicated in chromatin remodeling and sister chromatid recombination (16–19). Cells from H2AX-deficient mice are defective in DNA double-strand break repair and show chromosomal instability, but animals are not particularly prone to develop cancer unless p53 is absent (20–23). Phosphorylation of H2AX by c-jun-N-kinase has recently been implicated in the induction of apoptosis after UV irradiation (24).

All of these H2AX functions are associated with its nucleosomal localization. However, there is compelling evidence that histone proteins have activities outside their normal nucleosomal context. In yeast, a surveillance system has been identified that monitors the level of free, non-nucleosomal histone proteins that arise predominantly during S phase where newly synthesized histones need to be incorporated into nucleosomes behind the replication fork (25, 26). It is conceivable that such control mechanisms are particularly important following perturbations of replication fork progression (27). Failure to maintain a tight control over the pool of free histones can lead to excessive amounts of free histones and deleterious cellular consequences, including chromatin defects, abrogated transcription (28, 29), genomic instability, and impaired cell viability (25).

We report here that imatinib induces apoptosis in GIST cells in part through the up-regulation of free histone H2AX. We found that GIST cells can down-regulate H2AX protein levels in a
KIT-dependent manner, and that the imatinib-induced increase of H2AX protein levels sensitizes tumor cells to undergo cell death by blocking active gene transcription. Collectively, our results highlight the role of histone-mediated cytotoxicity in GIST cell death induced by imatinib and establish an unexpected role of H2AX in this process.

Materials and Methods

Cell culture and transfections. The human GIST cell line GIST882 was derived from an untreated metastatic GIST and maintained in RPMI 1640 supplemented with 15% fetal bovine serum (FBS: Mediatech, Herndon, VA), 1% t-glutamine, 50 units/mL penicillin (Cambrex, Walkersville, MD), and 50 μg/mL streptomycin (Cambrex) as described earlier (30). The GIST48 cell line was derived from an imatinib-resistant GIST and was maintained in Ham’s F-10 supplemented with 15% FBS, 1% t-glutamine, 50 units/mL penicillin (Cambrex), 50 μg/mL streptomycin (Cambrex), 30 μg/mL bovine pituitary extract (Fisher Scientific, Pittsburgh, PA), and 0.5% MTO+ Serum Extender (Fisher Scientific).

IMR90 non-neoplastic human lung fibroblasts were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to manufacturer's recommendations.

GIST882 cells were transiently transfected by nucleofection (Amaxa, Cologne, Germany). Briefly, cells were trypsinized, and 3 × 10⁶ cells per experiment were transfected with 5 μg of plasmid DNA.

NH₂-terminal green fluorescent protein (GFP) fusion plasmids encoding wild-type H2AX and H2AX in which Ser⁷⁸⁷ has been substituted with alanine (H2AX-S139A) were a generous gift of Joseph S. Siino (University of California Davis School of Medicine, Davis, CA) and Nicolai Tomilin (Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia; ref. 31). An empty pcMV-GFP vector was included as control in all experiments. A plasmid encoding myc-tagged H2A was a generous gift of4and was expressed in H2AX-S139A cells. Heterozygous KitV558D+/− mice. Heterozygous KitV558D+/− mice were generated as described by Sommer et al. (32) and treated with imatinib as reported by Rossi et al. (33). Briefly, mice selected for treatment had no apparent signs of disease and were 3 to 5 months old. The animals were reported by Rossi et al. (33) Briefly, mice selected for treatment had no apparent signs of disease and were 3 to 5 months old. The animals were treated with 45 mg/kg imatinib mesylate or placebo twice per day for a total of 24 h and were killed 6 h after the last administration of the drug. Tumors were harvested, fixed in 4% paraformaldehyde and embedded in paraffin for histology and immunohistochemistry.

Immunologic and cell staining methods. Protein lysates of cells growing as monolayer were prepared by scraping cells into radioimmunoprecipitation assay buffer (1% NP40, 50 mM/L Tris-HCl (pH 8.0), 100 mM/L sodium fluoride, 30 mM/L sodium PPI, 2 mM/L sodium molybdate, 5 mM/L EDTA, 2 mM/L sodium orthovanadate) containing protease inhibitors (10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM/L phenylmethylsulfonyl fluoride). Lysates were incubated for 1 h with shaking at 4°C and then cleared by centrifugation for 30 min at 14,000 rpm at 4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA). Thirty micrograms of protein were loaded on a 4% to 12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and blotted onto a nitrocellulose membrane.

For immunofluorescence analysis, cells grown on coverslips were briefly washed in PBS and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were then washed in PBS and permeabilized with 1% Triton X-100 in PBS for 15 min at room temperature followed by washing in PBS and blocking with 10% normal donkey serum (Jackson Immunoresearch, West Grove, PA) for 15 min at room temperature. Cells were then incubated with primary antibodies overnight at 4°C in a humid chamber and incubated for another 3 h at 37°C the next morning. After a brief wash in PBS, cells were incubated with FITC–anti-mouse secondary antibodies (Jackson Immunoresearch) for at least 2 h at 37°C, washed with PBS, and counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Cells were analyzed using an Olympus (Center Valley, PA) AX70 epifluorescence microscope equipped with a SpotRT digital camera.

For immunofluorescence analysis of paraffin sections, slides were baked at 65°C overnight, deparaffinized in xylene, and dehydrated in 100% ethanol. After rehydration in a graded ethanol series, slides were washed in distilled H₂O and microwave treated in 0.01 mol/L sodium citrate for a total of 30 min. Slides were cooled for at least 15 min and then washed in distilled H₂O and PBS. Blocking was carried out in 10% normal donkey serum for 30 min at room temperature. Incubations with primary and secondary antibodies were done as described above.

Primary antibodies used for immunoblotting and immunofluorescence were monoclonal phospho-H2AX S139 (Upstate, Charlottesville, VA), polyclonal phospho-H2AX S139 (Trevigen, Gaithersburg, MD), H2AX (Bethyl Laboratorys, Montgomery, TX), H2A (Cell Signaling, Beverly, MA), phospho-KIT Y703 (Zymed Laboratories, South San Francisco, CA), KIT (DakoCyto- tomation, Carpinteria, CA), PARP (Zymed Laboratorys), mono-ubiquitin (BD Biosciences PharMingen, San Jose, CA), myc-tag (Cell Signaling), ORC2 (BD Biosciences PharMingen), actin (Sigma, St. Louis, MO), poly-ubiquitated (S473) and total AKT (Cell Signaling), and phosphorylated (S2448) and total mTOR (Cell Signaling).

Apopotic cells were visualized using the In situ Cell Death Detection Kit (Roche Applied Sciences, Indianapolis, IN) according to manufacturer's instructions.

Inhibitor treatments. Cells were incubated in 1 μmol/L imatinib mesylate (kindly provided by Novartis) dissolved in DMSO or mock treated (0.1% DMSO) for up to 72 h. The caspase inhibitor Z-VAD-FMK (EMD Biosciences, San Diego, CA) was used at 100 μmol/L either alone or in combination with 1 μmol/L imatinib for 24 h. Other treatments included the proteasome inhibitor Z-LVVS (Biomol, Plymouth Meeting, PA) at 1 μmol/L, the phosphoinositide-3-kinase (PI3K) inhibitor LY294002 at increasing concentrations up to 25 μmol/L (EMD Biosciences) as indicated, the PIK inhibitor ZSTK774 at increasing concentrations up to 200 μmol/L (EMD Biosciences) as indicated, the mTOR inhibitor rapamycin at 20 μmol/L (EMD Biosciences) and the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 inhibitor U0126 at 10 μmol/L (EMD Biosciences), all in DMSO for 24 h. The RNA polymerase II inhibitor α-amanitin (Sigma) was reconstituted in distilled H₂O at 1 mg/mL and used at the indicated concentrations.

Small interfering RNA. Pooled synthetic RNA duplexes were used to reduce KIT or H2AX protein expression (siGENOME SmartPool, Dharmacon, Lafayette, CO, and Silencer, Ambion, Austin, TX, respectively). Briefly, GIST882 cells were trypsinized, and 3 × 10⁶ cells were transfected with 5 μL of 20 μmol/L annealed RNA duplexes using nucleofection (Amaxa). Cells were then transfected to 35-mm tissue culture dishes with 2 mL RPMI 1640 free of antibiotics. Experiments were done at the indicated time points.

Ubiquitination assay. To assess the ubiquitination of H2AX in GIST cells, proteins from GIST882 cells were extracted in 20 μmol/L Tris-HCl 0.25 mM/L EDTA containing 2 mM/L DTT, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. The suspension was spun at 10,000 × g for 10 min at 4°C, and the supernatant (S-10 fraction) was collected. Per ubiquitination reaction, 0.5 μg of recombinant human H2AX protein (Upstate) was incubated in GIST882 cell extract containing an ATP-regeneration system [50 μmol/L Tris-HCl (pH 7.4), 5 mM/L MgCl₂ 2 mM/L ATP 10 μmol/L phosphocreatine, 1.5 units/mL creatine phosphokinase], together with 1 μmol/L ubiquitin aldehyde, 100 μmol/L methyl ubiquitin (both Boston Biochem, Cambridge, MA), 1 μmol/L DTT, and 10 μmol/L okadaic acid. The reaction was carried out at 37°C for up to 90 min, and aliquots were taken at various time points. The reaction was stopped by adding SDS sample loading buffer and heating the samples to 95°C. Proteins were separated by SDS-gel electrophoresis and analyzed for ubiquitination of H2AX by staining with a mouse monoclonal antibody to mono-ubiquitin (BD Biosciences PharMingen).

Chromatin fractionation. Chromatin fractionation was done according to Mendez and Stillman (34). Briefly, cells were trypsinized, collected by centrifugation at 1,100 rpm for 10 min, and washed once with PBS. The cell pellet was resuspended in Buffer A [10 mM/L HEPES (pH 7.9), 10 mM/L KCl, 1.5 mM/L MgCl₂, 10 mM/L NaF, 340 mM/L sucrose, 10% glycerol,
Results

Imatinib induces a delayed apoptotic response. To determine the kinetics of GIST cell apoptosis following imatinib treatment, we used the human GIST cell line GIST882, which carries a constitutively activating homozygous KIT mutation in exon 13 (K642E) corresponding to the first lobe of the split kinase domain (30). GIST882 is one of only a few GIST cell lines currently available that are sensitive to imatinib. Cells were treated with 1 μmol/L imatinib or mock treated with 0.1% DMSO for up to 72 h, and the frequency of apoptotic cells was measured by terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) staining (Fig. 1A). Although an increase of apoptosis became detectable after 24 h from 0.6% in controls to 5% in imatinib-treated populations (P ≤ 0.01), a major effect on cell viability was not observed until 72 h with an increase of apoptotic cells from 0.5% in controls to 61.4% in treated populations (P ≤ 0.0001; Fig. 1B). These results show that the apoptotic response of GIST cells to imatinib is time dependent, and that a major increase of cell death occurs with a considerable delay after onset of treatment.

Imatinib induces an up-regulation of soluble histone H2AX in GIST cells. Histone H2AX has previously been implicated in cell death following cytotoxic therapy (35). To explore mechanisms of imatinib-induced tumor cell apoptosis, we analyzed γ-H2AX expression in imatinib and mock-treated GIST882 cells by immunoblotting. Imatinib induced a massive increase of γ-H2AX levels at 72 h, which was already evident at 8 h (Fig. 2A). Unexpectedly, this imatinib-induced γ-H2AX overexpression was accompanied by an increase in total H2AX protein levels (Fig. 2A). Remarkably, no increase of core histone H2A was found in imatinib-treated cells (Fig. 2B).

The fact that experiments were done using whole cell extracts and a standard lysis buffer without acid histone extraction prompted us to investigate whether the increase of γ-H2AX and H2AX was due to soluble H2AX proteins. We obtained subcellular fractions (S2, cytoplasmic; S3, soluble nuclear; P3, chromatin; ref. 34) from imatinib- and mock-treated GIST882 cells. Whereas in mock-treated cells, both γ-H2AX and H2AX were detected only in the P3 fraction by immunoblotting, imatinib-treated cells showed mislocalization of both γ-H2AX and H2AX to soluble cytoplasmic, and to a lesser extent, nuclear fractions (Fig. 2C), suggesting that mostly soluble H2AX accounts for the massive increase shown in Fig. 2A.

The up-regulation of soluble H2AX in imatinib-treated GIST882 was causally related to inhibition of the KIT oncoprotein because an increase of γ-H2AX and H2AX was seen in GIST cells after KIT knockdown by small interfering RNA (siRNA; Fig. 2D). Moreover, H2AX protein expression was not detected in tissue samples obtained from untreated primary human GISTs with known activating mutations of the KIT oncoprotein (data not shown).

We next explored whether imatinib-induced H2AX up-regulation is due to increased protein stability. The ubiquitin-proteasome machinery represents the major pathway that regulates protein turnover in cells (36). When GIST882 and non-neoplastic human fibroblasts (IMR90) were treated with a proteasome inhibitor, increased γ-H2AX and H2AX levels were detected in GIST882 cells.
but not in IMR90 cells (Fig. 2E). Because these results suggest that H2AX is degraded by the ubiquitin-proteasome system, we tested whether H2AX becomes polyubiquitinated in GIST cells. An in vitro ubiquitination assay using recombinant human H2AX as substrate showed that GIST882 cell lysate promotes the polyubiquitination of human recombinant H2AX (Fig. 2F). These results suggest that oncogenic KIT decreases H2AX levels through stimulation of its ubiquitin-mediated degradation and is in line with findings that H2AX mRNA levels were not up-regulated in a GIST mouse model after imatinib treatment.7

Signaling events responsible for the up-regulation of H2AX levels were evaluated by treating GIST882 cells with other small-molecule inhibitors that target protein kinases downstream of KIT. The PI3K inhibitor LY294002 induced γ-H2AX and H2AX levels similar to imatinib, whereas the MEK1/2 inhibitor U0126 did not (Fig. 2G). Treatment of GIST882 cells with ZSTK474, a PI3K inhibitor that binds to different amino acid sites in the ATP-binding pocket of PI3K than LY294002 (37), likewise led to an increase of γ-H2AX and H2AX (Supplementary Fig. S1A). The mTOR inhibitor rapamycin did not trigger an increase of γ-H2AX and H2AX levels.

LY294002 is not a specific inhibitor of PI3K and, at the concentration above, equally inhibits PI3K and mTOR (38). mTOR exists in two functionally distinct complexes in mammalian cells, mTOR complex 1 (mTORC1) and mTORC2 (39). Rapamycin only partially inhibits mTORC1, whereas LY294002 inhibits both, mTORC1 and mTORC2. To investigate the role of PI3K in comparison to mTOR inhibition in more detail, we did a dose-response analysis of GIST882 cells treated with increasing concentrations of LY294002. We found up-regulation of γ-H2AX and H2AX at concentrations that led to reduced levels of phospho-AKT and at the same time a reduced phosphorylation of mTOR at

7 P. Besmer, personal communication.
GIST882 cells were transiently transfected with GFP-tagged H2AX to establish proapoptotic activities. First and most importantly, ectopic up-regulation of H2AX is a cause and not merely a consequence of apoptosis. To determine whether H2AX up-regulation correlates with imatinib sensitivity in GIST, we compared H2AX induction in imatinib-sensitive cells (GIST882) versus a cell line derived from a clinically resistant GIST (GIST48) with a secondary KIT mutation in exon 17 (D820A) conferring imatinib resistance. GIST48 cells did not show H2AX up-regulation after imatinib treatment (Fig. 2H). However, inhibition of the PI3K pathway with LY294002 led to a significant increase of apoptotic cells in imatinib-resistant GIST48 compared with cells treated with DMSO or imatinib (data not shown).

The up-regulation of soluble H2AX in GIST cells is a cause and not a consequence of apoptosis. A release of free histone proteins into the cytoplasm has been previously reported to occur during apoptosis (41). Although our finding that core histone H2A does not increase following imatinib treatment (Fig. 2B) suggests that the up-regulation of H2AX is not due to a nonspecific release of histone proteins, we did a series of additional experiments to establish that H2AX up-regulation is a cause and not merely a consequence of apoptosis.

Several lines of evidence support our hypothesis that H2AX has direct proapoptotic activities. First and most importantly, ectopic expression of H2AX was found to stimulate enhanced cell death. GIST882 cells were transiently transfected with GFP-tagged H2AX or mutated H2AX in which Ser139 has been replaced by alanine (H2AX-S139A; ref. 31). An immunoblot analysis of subcellular fractions confirmed that transient transfection of H2AX or H2AX-S139A results in free, non-nucleosomal histone proteins (data not shown). Transfected cells were identified by GFP positivity, and apoptotic cells were assessed based on nuclear morphology and TUNEL staining (Fig. 3A). A 16.4-fold increase of apoptotic cells was detected between empty GFP vector-transfected controls (1.6%) and H2AX-transfected cells (26.2%; P < 0.005) at 24 h, and a 4.5-fold increase of apoptotic cells from 8.9% in empty vector controls to 39.8% in H2AX-transfected cells at 48 h (P < 0.005; Fig. 3B). This increase in apoptotic cells coincided with caspase-3 cleavage as assessed by immunoblotting (Fig. 3C). Transfection of cells with H2AX-S139A still caused a significant increase of apoptotic cells at both time points (8.1% at 24 h and 19.6% at 48 h; P < 0.05), albeit to a lesser extent than wild-type H2AX, suggesting that proapoptotic functions of H2AX in GIST cells rely in only part on activities associated with phosphorylation of H2AX at Ser139.

Transfection of GIST cells with core histone H2A did not cause enhanced apoptosis when compared with cells transfected with empty control vector (Fig. 3D and E). Second, we found that the caspase inhibitor Z-VAD-FMK effectively inhibited apoptosis as measured by poly-(ADP-ribose)-polymerase (PARP) cleavage in imatinib-treated GIST882 cells, but had no effect on the imatinib-induced up-regulation of H2AX expression (Fig. 3F). This finding stands in contrast to a previous report (41), in which the apoptotic release of free histone proteins into the cytoplasm was blocked by Z-VAD-FMK, hence further supporting our hypothesis that in our model, H2AX up-regulation is not simply a consequence of apoptosis.
obtained after 24 h of imatinib therapy and analyzed for
were treated with imatinib or placebo (33). Tumor samples were
treated GIST882 cells (Fig. 4A). GIST882 cells were cotransfected
with DsRED as a transfection marker and analyzed for apoptotic
morphology (Fig. 4B). A 2.2-fold increase of surviving cells was
detected in imatinib-treated cell populations transfected with
H2AX siRNAs compared with cells transfected with control siRNA
(P < 0.0001) and 6% in H2AX-S139A–expressing cells (P < 0.05; Fig. 6B). After 48 h, a major increase of
cells with chromatin aggregation was detected from 0% in controls
to 28.8% in H2AX-expressing cells (P < 0.01), whereas the increase in
H2AX-S139A-expressing cells to 8.9% was less pronounced albeit
still significant in comparison to controls (P < 0.05).

Chromatin aggregation induced by excess histone has previously
been shown to abrogate transcription (28). We therefore
determined whether increased H2AX levels could interfere with ongoing
transcription using a nuclear runoff assay. In vitro transcription
from a CMV immediate early promoter was carried out in the
presence of increasing amounts of recombinant H2AX protein. A
dose-dependent decrease of the 363-nucleotide runoff transcript
was detected when increasing amounts of H2AX were added to the
reaction (Fig. 6C). These findings clearly indicate that excess H2AX
can block ongoing gene transcription.

To directly analyze whether abrogation of gene transcription
stimulates apoptosis in GIST cells, we treated GIST882 cells with
α-amanitin, a specific inhibitor of RNA polymerase II (pol II). This

imatinib-treated animals also showed an altered nuclear morpho-
logy, most importantly chromatin aggregation (Fig. 5B).

Overexpression of H2AX causes a transcriptional block that
sensitizes GIST cells to apoptosis. Given the chromatin
alterations detected in GISTs of imatinib-treated Kit<sup>V558D</sup>/+ mice,
we sought to determine whether H2AX overexpression directly
induces changes of chromatin structure. A morphologic analysis of
GIST882 cells transiently transfected with H2AX revealed pheno-
typical changes that mimicked the chromatin aggregation detected
in mice, whereas such alterations were absent in cells transfected
with an empty GFP vector (Fig. 6A). Quantification of the
proportion of cells with chromatin aggregation showed a significant
increase after 24 h from 0% in empty vector controls to 8.9% in
H2AX-expressing cells (P < 0.0001) and 6% in H2AX-S139A–
expressing cells (P < 0.05; Fig. 6B). After 48 h, a major increase of
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Contributions of H2AX to imatinib-induced GIST cell death were
determined by siRNA-mediated knockdown of H2AX in imatinib-
treated GIST882 cells (Fig. 4A). GIST882 cells were cotransfected
with DsRED as a transfection marker and analyzed for apoptotic
morphology (Fig. 4B). A 2.2-fold increase of surviving cells was
detected in imatinib-treated cell populations transfected with
H2AX siRNAs compared with cells transfected with control siRNA
(P ≤ 0.01; Fig. 4C), indicating that up-regulation of H2AX is in part
involved in imatinib-induced apoptosis in GIST cells.

Collectively, these results suggest that the apoptotic effect of
imatinib on tumor cells expressing tyrosine kinase oncoproteins is
channeled, at least in part, through the induction of excessive levels
of soluble H2AX.

Up-regulation of γ-H2AX in a GIST mouse model following
treatment with imatinib. To determine the in vivo relevance of
γ-H2AX up-regulation following imatinib treatment of GISTs, we
used a GIST mouse model in which animals that carry a
heterozygous Kit exon 11 activating mutation (Kit<sup>V558D</sup>+/−; ref. 32)
were treated with imatinib or placebo (33). Tumor samples were
obtained after 24 h of imatinib therapy and analyzed for γ-H2AX
by immunofluorescence microscopy (Fig. 5). Scattered γ-H2AX–
positive tumor cells were readily detectable in imatinib-treated
animals but not in mock-treated mice (Fig. 5A) and corresponded
well with the amount of cells positive for cleaved caspase-3 (33).
Remarkably, many tumor cells that were positive for γ-H2AX in

Figure 4. Depletion of H2AX attenuates the apoptotic response of GIST cells
to imatinib. A, immunoblot analysis of GIST882 cells transfected with control
siRNA duplexes and siRNA targeting H2AX after simultaneous treatment with
1 μmol/L imatinib for 72 h. B, fluorescence microscopic analysis of GIST882 cells
transfected with siRNA duplexes targeting H2AX and simultaneous treatment
with 1 μmol/L imatinib for 72 h. DsRED was used as transfection marker. Note
the apoptotic fragmentation of the nuclei in the nontransfected cells and the
normal nuclear morphology in the H2AX siRNA-transfected cells. Bar, 50 μm.
C, quantification of surviving GIST882 cells after transfection with either control
siRNA or siRNAs targeting H2AX and simultaneous treatment with 1 μmol/L
imatinib for 72 h. Columns, mean of at least three independent experiments
with at least 100 cells counted per experiment; bars, SE.

Figure 5. In vivo up-regulation of H2AX following imatinib treatment.
A, histomorphologic (H&E) and immunofluorescence analysis of GISTs in
Kit<sup>V558D</sup>+/− mice after either 24 h placebo treatment or treatment with imatinib.
Note the scattered γ-H2AX–positive tumor cells following imatinib treatment.
Nuclei stained with DAPI. Bar, 100 μm. B, high-power magnification of
γ-H2AX–positive rodent GIST cells after imatinib treatment. Note the
γ-H2AX–positive tumor cell with chromatin aggregation (arrow). Nuclei
stained with DAPI. Bar, 10 μm.
inhibitor has been suggested to interact with the largest subunit of RNA pol II, Rpb1, to inhibit transcription initiation and elongation of mRNAs (42). We found that α-amanitin selectively induced GIST cell apoptosis whereas it had no or little effect in IMR90 normal human fibroblasts (Fig. 6D). Statistically highly significant increases (P ≤ 0.0001) of apoptotic GIST882 cells were detected after 48 h treatment with α-amanitin at all concentrations used ranging from 0.1 to 5 μg/mL in comparison to IMR90 cells. Remarkably, 0.5 and 1 μg/mL α-amanitin led to 16.9% and 39.8% of apoptotic GIST cells with virtually no toxicity in normal IMR90 fibroblasts (0% and 0.35%, respectively; Fig. 6D). α-Amanitin was active in both cell populations as evidenced by a reduced expression of the initiating form of RNA polymerase II (Ser5 phosphorylated) by immunoblotting (data not shown).

Taken together, our results suggest that overexpression of soluble histone H2AX sensitizes GIST cells to apoptosis by abrogating ongoing gene transcription.

Discussion

Here, we show that the proapoptotic activity of imatinib in GIST cells involves an up-regulation of soluble histone H2AX. Integrity of the COOH-terminal SQE motif of H2AX is not strictly required but may enhance this activity because mutant H2AX-S139A was less efficient in triggering cell death than wild-type H2AX. We propose a model in which excessive levels of free histone H2AX cause chromatin aggregation, which leads to impaired transcription (28) and thereby sensitizes tumor cells to undergo apoptosis. GIST cells seem to be particularly sensitive to reduced RNA pol II transcription as shown here (Fig. 6D) and in a recent study (43). The fact that depletion of H2AX protein by siRNA did not completely prevent imatinib-induced cell death (Fig. 3) may be related to an incomplete protein knockdown. However, given that KIT activates several downstream signaling pathways, it is possible that more than one mechanism contributes to GIST cell death. Imatinib has been reported to modulate proapoptotic signaling pathways in chronic myelogenous leukemia cells (44), and a recent study suggests that imatinib can cause death of nontumor cells by inducing endoplasmic reticulum stress (45).

Our finding that GIST cells can down-regulate H2AX in a pathway that involves PI3K, mTOR, and the ubiquitin/proteasome machinery lends important support to the relevance of H2AX as a tumor suppressor because it is, to the best of our knowledge, the first demonstration of its dynamic regulation in human tumor cells. Moreover, these results may have implications for a broad range of tumors that carry PI3K mutations (46) and underscore the importance of PI3K and mTOR as drug targets (6, 47).

There are several scenarios that may explain why tumor cells with constitutively activated protein kinases down-regulate H2AX. The continuous proliferative signal of an oncogenically activated kinase is likely to trigger unscheduled S phase entry and, thus, an increased stalling of replication forks (48, 49). This DNA replication stress may result in a frequent uncoupling of H2AX synthesis from its incorporation into nucleosomes (50). Results in yeast show that these events lead to excess soluble histone proteins, which is associated with a remarkable cellular toxicity (25). Because accumulation of free H2AX stimulates apoptosis as shown in the present study, its down-regulation would allow tumor cells to escape from cell death. Moreover, given the role of H2AX as a critical nexus between chromatin and DNA damage signaling cascades, an overall reduction of H2AX levels in chromatin due to rapid degradation and decreased incorporation into nucleosomes might represent an efficient way to abrogate growth-inhibitory mechanisms associated with DNA damage. This idea is consistent with the clinical observation that tumors expressing activated tyrosine kinase oncoproteins are frequently resistant to DNA damage-dependent apoptosis induced by cytotoxic chemotherapy or irradiation (51).

Our results have various implications for cancer therapy. First, the observation that H2AX up-regulation is critical for GIST cell sensitivity to imatinib suggests novel therapeutic approaches in which H2AX induction might be accomplished by alternative mechanisms, such as proteasome inhibition, thereby countering imatinib resistance. Second, the observation that PI3K inhibition leads to induction of H2AX expression provides a novel mechanistic
basis for antiapoptotic roles of P3K and suggests that P3K is a promising therapeutic target in GIST, either combined with KIT inhibition or alone (47). Third, our findings suggest that H2AX upregulation by tyrosine kinase oncoprotein inhibitors may restore tumor sensitivity to conventional chemo- or radiotherapy.

Further understanding of how oncoprotein gene kinases overcome anticancer barriers during tumor evolution is likely to improve the therapeutic and preventive use of targeted small-molecule inhibitors.

Acknowledgments

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Ying Liu, Michelle Tseng, Sophie A. Perdreau, et al.


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