Ras Induces Chromosome Instability and Abrogation of the DNA Damage Response

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Abstract

Ras mutations are frequent in thyroid tumors, the most common endocrine malignancy. The ability of Ras to transform thyroid cells is thought to rely on its mitogenic activity. Unexpectedly, acute expression of activated Ras in normal rat thyroid cells induced a DNA damage response, followed by apoptosis. Notably, a subpopulation of cells evaded apoptosis and emerged with features of transformation, including the loss of epithelial morphology, dedifferentiation, and the acquisition of hormone- and anchorage-independent proliferation. Strikingly, the surviving cells showed marked chromosomal instability. Acutely, Ras stimulated replication stress as evidenced by the induction of ataxia telangectasia mutated and Rad3-related protein kinase (ATR) activity (Chk1 phosphorylation) and of γH2AX, a marker of DNA damage. Despite the activation of a checkpoint, cells continued through mitosis in the face of DNA damage, resulting in an increase in cells harboring micronuclei, an indication of defects in chromosome segregation and other forms of chromosome damage. Cells that survived exposure to Ras continued to exhibit replication stress (ATR activation) but no longer exhibited γH2AX or full activation of p53. When rechallenged with Ras or DNA-damaging agents, the surviving cells were more resistant to apoptosis than parental cells. These data show that acute expression of activated Ras is sufficient to induce chromosomal instability in the absence of other signals, and suggest that Ras-induced chromosomal instability arises as a consequence of defects in the processing of DNA damage. Hence, abrogation of the DNA damage response may constitute a novel mechanism for Ras transformation. (Cancer Res 2006; 66(21): 10505-12)

Introduction

The contribution of Ras to neoplastic transformation has been difficult to ascertain given the pleiotropic responses induced by Ras. A large body of evidence exists showing that expression of activated Ras in established cell lines stimulates cell proliferation, induces apoptosis, and abolishes differentiation. There is emerging evidence that Ras stimulates genome destabilization. Human thyroid tumors exhibit frequent activating mutations in Ras and B-Raf, genetic evidence that Ras-dependent signaling is a major contributor to thyroid cell transformation (1, 2). Retroviral-mediated transduction of activated Ras into primary human thyrocytes enhanced proliferation (3). Similarly, acute microinjection of activated Ras protein stimulated DNA synthesis in human (4) as well as in rat (5, 6) thyroid cells. Paradoxically, when expressed acutely in normal rat thyroid cells, Ras stimulated apoptosis (7, 8), an effect inconsistent with the ability of Ras to stimulate DNA synthesis in microinjection assays and the high frequency of Ras mutations in thyroid tumors. Our findings reconcile these seemingly disparate reports. We show that sustained mitogenic signaling induced by Ras provokes a checkpoint response that induces apoptosis. Significantly, in the absence of overt selection for Ras, cells survive apoptosis and emerge with features of transformation, including alterations in growth regulation and differentiated status. Strikingly, the surviving cells exhibit widespread chromosomal instability. The ability of Ras to induce chromosomal destabilization provides a facile mechanism for the elaboration of widespread genetic changes that could enhance the survival of cells harboring Ras mutations and contribute to the acquisition of the transformed phenotype.

Although a great deal is known regarding other mechanisms of Ras transformation, quite little is known about the mechanism through which Ras induces genome destabilization. Activated Ras induced chromosome instability within one to two cell cycles following its expression in p53-deficient fibroblasts (9, 10). Together, Ras and E1A transformed p53-null mouse embryonic fibroblasts, giving rise to cells that were markedly aneuploid (11). Expression of K-Ras from its endogenous promoter induced tetraploidy in primary murine embryonic fibroblasts, suggesting that modest expression of activated Ras is capable of inducing chromosome instability in the presence of wild-type p53 (12). Our data provide new insight into the mechanism through which Ras induces chromosomal instability. We present evidence that suggests that chromosomal instability arises from defects in the recognition and/or processing of DNA damage invoked by Ras. Acutely, Ras provokes a checkpoint response, characterized by the induction of γH2AX and activation of the ataxia telangectasia mutated (ATM) and Rad3-related protein kinase (ATR). ATR is a member of a family of phosphatidylinositol 3-kinase–like kinases that also includes ATM and DNA-dependent protein kinase. ATR is an essential gene whose product plays a critical role in normal cell cycle progression as well as in the cellular response to aberrations in DNA replication and/or repair (13). ATR activity is transmitted through a variety of downstream effectors, including Chk1 and p53. Surprisingly, although cells that survive Ras exposure exhibit checkpoint activation, they continue to cycle and are attenuated in their response to DNA damage. As a consequence, these cells show marked chromosomal instability, are resistant to apoptosis, and acquire features of transformation.

Materials and Methods

Antibodies and reagents. Phosphorylated Chk1 (Ser345), p53 (Ser15), and extracellular signal-regulated kinase (ERK) antibodies were purchased from Cell Signaling (Beverly, MA). MPM-2 and phosphorylated H2AX (Ser139) antibodies were from Upstate (Charlottesville, NY). Antibodies to ERK2 and...
β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Phycocerythrin-conjugated Annexin V was from Caltag Laboratories (San Francisco, CA), bromodeoxyuridine (BrdU/rd) antibody was from Bionesian (Sacramento, CA), and thyroglobulin antibody was from DAKO (Glostrup, Denmark). Etoposide was obtained from Sigma (St. Louis, MO).

**Cell culture and adenoviral infection.** Wistar rat thyroid (WRT) cells were propagated in Coon’s modified Ham’s F12 medium supplemented with bovine TSH (1 μU/mL), insulin (10 μg/mL), calf serum (5%), and transferrin (5 μg/mL) as previously described (14). Cells were grown to 70% confluence and rendered quiescent by starvation in TSH, growth factor, and serum-free basal medium for 48 to 72 hours. Hemagglutinin-tagged wild-type and activated (V12) H-Ras adenoviruses were constructed using the AdEasy Vector System (Q-Biogene, Carlsbad, CA). H-Ras/L61 adenovirus was a generous gift from Dr. J. Nevin (Howard Hughes Medical Institute, Duke University). Adenoviruses were propagated in QBI-293 cells, purified by CsCl centrifugation, and stored at −80 °C. Quiescent cells were infected with adenoviruses overnight and maintained in basal medium thereafter. The morning after infection is called day 1 postinfection (16 hours after addition of virus). Ras/L61 adenovirus was infected at a multiplicity of infection (MOI) of 5 infectious units/cell (IU/cell, as determined by titration in 293 cells) and RasV12 at MOI of 3.5 IU/cell, conditions where >95% of the cells were infected. RasV12-transformed WRT cells (15, 16) were propagated in the same medium as WRT cells, with the addition of genetin (150 μg/mL).

**Isolation of cells surviving Ras infection.** Two approaches were used to isolate cells that survived exposure to Ras. In the first, floating cells were collected at day 4 postinfection, plated in growth medium overnight (to allow attachment), and then transferred to TSH-deficient medium for propagation. Individual plates of infected cells were collected as pools of surviving cells. Four independent infections harvested in this manner gave rise to pools 4, 5, 6, and 8. In the second approach, Ras-infected cells were maintained in basal medium with daily medium changes until no floating cells were observed. The remaining adherent cells were transferred to TSH-deficient medium (pool 1) or serum-supplemented basal medium (pool 2). Control infections with adenoviruses expressing RasG12 or LacZ did not give rise to colonies capable of TSH-independent proliferation. Although these cells did not undergo apoptosis, we were unable to expand cells from these infections in the absence of TSH. Hence, the isolation of surviving cells capable of TSH-independent proliferation requires acute exposure to activated Ras. Once the pools of cells that survived RasL61 or RasV12 infection reached 50% confluence, they were subcultured into two dishes, one of which was subsequently frozen and the other expanded for analysis.

**Western blotting.** Cells were harvested on ice in RIPA buffer containing protease and phosphatase inhibitors as described in ref. 17. Protein determinations were made and equal protein contents were analyzed by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blotting. Primary and secondary antibodies were used as recommended by the manufacturer. Proteins were detected using enhanced chemiluminescence and the FujiFilm LAS-3000 imaging system.

**DNA synthesis assessed by BrdU incorporation.** Cells plated on glass coverslips were starved for 48 hours in basal medium and labeled with BrdUrd for 4 hours. Cells were fixed in 3.7% formaldehyde/PBS for 20 minutes at room temperature and incubated with anti-BrdUrd antibody (1:100) in PBS containing 5 mg/mL bovine serum albumin, 0.5% NP40, 0.14 units/mL DNease, followed by FITC-conjugated anti-sheep IgG (1:200) in PBS. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Images were observed using a Zeiss Axioscope epifluorescence microscope and collected using Hamamatsu ORCA-ER digital camera and Axiovision 4.2 software.

**Fluorescence-activated cell sorting analysis for DNA content.** Floating and adherent cells were collected, fixed in ice cold MeOH for 30 minutes, and stained with propidium iodide (0.1 mg/mL) in RNase (100 units/mL) for 30 minutes in the dark as described in ref. (18). Cells were analyzed on BD Biosciences FACSCalibur using CellQuest Pro for cell cycle position and Modfit LT 3.1 for DNA index (DI).

**Annexin V staining.** Cells were suspended in binding buffer [0.8% NaCl, 0.037% CaCl2, 0.24% HEPES (pH 7.4)] and stained with phycoerythrin-conjugated anti-Annexin V (1:25) in binding buffer for 15 minutes at room temperature in the dark. Cells were washed and resuspended in 500 μL binding buffer and immediately analyzed by FACS Calibur using CellQuest Pro software.

**Growth curve assays.** Replicate plates of cells were plated overnight at 1 × 104 per dish in TSH-deficient growth medium. Duplicate plates were collected by trypsinization and counted using a hemocytometer.

**Anchorage-independent growth.** Cells were suspended in TSH-deficient growth medium containing 0.33% agar, and 2 × 104 cells were plated in duplicate on a bottom layer of 0.45% agar. Cells were fed 200 μL medium once per week. After 20 days, colonies were stained in 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) overnight.

**Analysis of chromosome number.** Cells were incubated in growth medium containing 0.1 μmol/L nocodazole for 2 hours, harvested by trypsinization, centrifuged, and resuspended in 75 mmol/L KCl in PBS. After incubation for 20 minutes at 37 °C, cells were prefixed by adding 1:10 volume of methanol (MeOH)/acetic acid (3:1) for 1 minute at room temperature, collected by centrifugation, resuspended in fresh fixative, and incubated for a further 10 minutes. Cells were dropped onto cold slides, dried overnight, and stained with DAPI.

**Results**

**Acute expression of Ras induces a DNA damage checkpoint.** As reported previously (7), acute expression of activated Ras (H-Ras/L61, hereafter called Ras) induced quiescent WRT cells to enter the cell cycle. Ras-infected cells entered S phase (Fig. 1A). down arrow within 40 hours (day 2) postinfection, and either arrested there or progressed only slowly through the cell cycle. Notably, the proportion of G1-phase cells declined over time, indicating that very few cells completed the cell cycle and reentered G1 phase. Indeed, the proportion of apoptotic cells, as indicated by the presence of cells with hypodiploid DNA content (Fig. 1A, bracket) or cell surface expression of Annexin V, a marker of early apoptosis (Fig. 1C), increased beginning at day 3 postinfection. These results indicate that acutely, Ras induces deregulation of the cell cycle, an event that is accompanied by apoptosis.

The delayed transit of Ras-expressing cells through the cell cycle prompted us to investigate whether Ras activated cell cycle checkpoints. Hence, the effects of Ras on ATR, a key component of the replication checkpoint (19), were examined. Ras stimulated the activating phosphorylation of Chk1 on serine 345, an indicator of ATR activity, together with p53 phosphorylation on serine 15, a site phosphorylated by ATM and ATR (Fig. 1B). ATM and ATR are induced in response to replication stress and DNA double-strand breaks (19). Intriguingly, Ras stimulated serine 139 phosphorylation of the histone H2AX variant generating γH2AX (Fig. 1B), a modification that marks sites of DNA double-strand breaks undergoing repair (20). The induction of γH2AX and ATR activity preceded our ability to detect apoptotic cells, suggesting that thyroid cells respond acutely to Ras with DNA damage and checkpoint activation, followed by apoptosis. This response was difficult to reconcile with the high frequency of Ras mutation in human thyroid tumors; hence, we examined whether a subpopulation of cells survived exposure to Ras.

**Ras induces bypass of cell cycle checkpoints.** To determine whether a subpopulation of cells bypassed checkpoint activation, Ras-infected cells were examined for markers of mitosis. Two markers of M-phase cells, phosphorylation of histone H3 on serine 10 (21), and MPM2 reactivity, an indicator of cyclin B1/CDK-1 activity (22, 23), were analyzed. Intriguingly, Ras stimulated histone H3 phosphorylation and MPM2 reactivity beginning at day 2 postinfection (Fig. 2A, B), a time that corresponds to the maximal increase in Ras activity as assessed.
by ERK activation. Both markers increased over time, suggesting continued entry of cells into M phase. The sustained induction of histone H3 phosphorylation and MPM2 reactivity (Fig. 2A, B) suggested that cells bypass the replication checkpoint and proceed into mitosis with DNA damage. In support of this notion, Ras stimulated a time-dependent increase in the proportion of cells harboring micronuclei, an indicator of aberrant mitoses and chromosome damage (Fig. 2C; ref. 24). This response was specific to activated Ras, as cells expressing wild-type Ras (Fig. 2C) or LacZ (data not shown) failed to induce micronuclei formation. Only cells that had both an intact nucleus and micronuclei (Fig. 2C, inset) were scored to exclude cells that exhibited fragmented nuclei secondary to apoptosis. Based on the frequency of micronuclei formation (approaching 8%), a substantial proportion of Ras-infected cells continued through mitosis with DNA damage. As the quantitation of micronuclei was restricted to adherent cells, it is likely that a far greater number of cells bypass checkpoint activation.

Cells survive Ras-stimulated apoptosis and exhibit markers of transformation. We next assessed whether we could isolate surviving cells to investigate the mechanism through which Ras-expressing cells bypass checkpoint activation and circumvent apoptosis. Quiescent cells were infected with adenoviruses encoding activated Ras (H-RasV12 and H-RasL61; see Materials and Methods) at MOIs sufficient to achieve Ras expression in >95% of the cells (data not shown). Beginning at day 4 postinfection, floating cells were removed and the remaining adherent cells were propagated. Alternatively, detached cells were collected at day 4 postinfection, replated, and surviving cells expanded (see Materials and Methods). Because infections were done in the absence of all growth factors and serum, surviving cells were transferred to serum-supplemented medium (see Materials and Methods). It is important to note that surviving cells were isolated in the absence of overt selection for Ras. Regardless of the strategy used, multiple pools of surviving cells were isolated from independent infections with RasL61- or RasV12-expressing adenoviruses. These pools are hereafter called Ras survivors or rs cells.

The phenotype of rs cells was examined beginning at the earliest passage (passage 2-3) when sufficient cells were available for analysis. Rs cells exhibited dramatic alterations in cell shape...
compared with parental WRT cells, which exhibit a characteristic epithelial morphology (Fig. 3A). Rs cells were spindle-shaped and exhibited loss of cell-cell contact, characteristics similar to those of thyroid cells stably expressing RasV12 (referred to hereafter as Ras-transformed cells or Ras stable in Fig. 3A; refs. 15, 16). WRT cells exhibit TSH-dependent proliferation. As the rs pools were expanded in the absence of TSH, the growth properties of these cells were clearly altered. Growth curve experiments documented that the ability of rs cells to proliferate in TSH-deficient growth medium was enhanced compared with WRT cells (Fig. 3B). Basal levels of DNA synthesis, as measured by BrdUrd incorporation in the absence of serum and growth factors, were markedly elevated in rs cells (30 ± 0.6% BrdUrd-positive rs 1 cells, 35 ± 4% BrdUrd-positive rs 2 cells) compared with WRT cells (1.5 ± 0.5% BrdUrd-positive cells). Furthermore, unlike WRT cells, rs cells exhibited anchorage-independent proliferation, although to varying extents (Fig. 3C).

In addition to alterations in cell shape and growth regulation, cell transformation is frequently associated with dedifferentiation. Thyroid cells express a panel of tissue-specific gene products, including thyroglobulin, a precursor to thyroid hormone. Thyroglobulin expression is regulated by TSH and insulin, and hence is extinguished when cells are transferred to hormone- and growth factor–deficient basal medium. The ability of forskolin/insulin (or TSH/insulin, data not shown) to induce thyroglobulin expression was abolished in the five pools of rs cells analyzed (rs 4 shown in Fig. 3D), results similar to those seen in RasV12-transformed WRT cells (14). Collectively, these data indicate that acute exposure of thyroid cells to activated Ras is sufficient to facilitate the emergence of cells with features of transformation.

Ras survivors exhibit chromosome instability. Genome destabilization could explain the rapid emergence of cells with features of transformation in the absence of overt selection for Ras. To explore whether Ras induced chromosomal instability, the DI of the rs pools was analyzed. Cells were harvested and stained with propidium iodide, and DNA content was analyzed by flow cytometry using Modfit, a program that uses an algorithm to detect cells with normal versus abnormal DNA content. Parental WRT cells were used to assign fluorescence intensities that correspond to diploid G1 (<50; Fig. 4A, top) and G2 (<100) DNA peaks, or a DI of 1.0. Compared with WRT cells, a proportion of rs 8 cells exhibited a rightward shift in the position of the G1 DNA peak (Fig. 4A, light gray curve, middle), indicating the presence of cells with increased G1 DNA content (DI = 1.13) compared with diploid cells (dark gray curve). To validate the significance of the observed shift, mixing experiments were done. Rs 8 cells were added to WRT cells (1:3 ratio) before staining and analysis. These experiments confirmed the existence of right-shifted G1 DNA peaks (Fig. 4A, bottom). Two additional rs pools and RasV12-transformed WRT cells exhibited an increase in DNA index (data not shown).

To further investigate the effects of Ras on chromosomal instability, chromosome number was assessed in metaphase spreads prepared from rs pools, WRT cells, and Ras-transformed WRT cells. Representative metaphase spreads are shown in Fig. 4D. The vast majority of WRT cells, ~80%, exhibited a modal chromosome number of 42 (Fig. 4B, C). On the other hand, rs cells and Ras-transformed WRT cells exhibited a wide variation in chromosome number (Fig. 4B), with <25% of cells containing 42 chromosomes (Fig. 4C). These data indicate that chromosomal instability is an early response to activated Ras. The continued presence of chromosomal instability in Ras-transformed cells suggests that genomic instability contributes to the maintenance of Ras transformation.

Altered response to DNA damage in cells that survive Ras expression. Rather than representing a consequence of neoplastic progression as was once believed, DNA damage seems to be an early event in malignant transformation. Precancerous lesions in humans exhibit a DNA damage response exemplified by ATR activation and the induction of γH2A.X (25, 26). Remarkably, ATR activation, assessed by Chk1 and p53 phosphorylation, was retained in rs cells (Fig. 5A). These data strongly suggest that the surviving cells are derived from the same population of cells in which Ras acutely induced a checkpoint response. The ability of the rs cells to proliferate in the presence of sustained checkpoint activation implied that these cells no longer respond appropriately to replication stress. In support of this notion, γH2A.X was not

Figure 3. Ras survivors exhibit a transformed phenotype. A, photomicrographs of growing WRT cells, Ras survivors (rs 1, 4, 5, 8), and Ras-transformed WRT cells (Ras stable). B, WRT and rs pools were plated and cell number was determined after 1, 3, and 6 days in TSH-deficient growth medium (see Materials and Methods). Points, mean summarized from three independent experiments; bars, SE. C, WRT and rs cells were plated in TSH-deficient medium containing soft agar and allowed to grow for 3 weeks. Colonies were stained with MTT and photographed (bottom, higher magnification). Three independent experiments were done for six pools of rs cells. D, WRT and rs cells were starved in basal medium for 48 hours, stimulated with vehicle (basal) or forskolin (10 μM/L, fsk) and insulin (10 μg/mL, ins) for 48 hours, and total cell protein was analyzed for thyroglobulin (TG) expression. Western blotting for actin confirmed equal protein loading. Three experiments were done for five rs pools with similar results.
Significantly, γH2AX was also absent from Ras-transformed WRT cells (Ras stable; Fig. 5B). Moreover, compared with cells acutely exposed to Ras (RasL61 in Fig. 5A), p53 was not fully activated in the rs cells. These data suggest that acute exposure to activated Ras selects for, or induces, alterations in the response to DNA damage, a factor that could contribute to the generation of chromosome instability.

Micronuclei were abundant in rs cells (Fig. 5C), consistent with the ability of these cells to bypass checkpoint activation in the presence of DNA damage. Basal levels of apoptosis were increased in rs cells compared with parental cells, suggesting that these cells are genetically unstable and give rise to cells with levels of DNA damage that are incompatible with survival (Fig. 5D). Compared with WRT cells acutely exposed to Ras, apoptosis was suppressed in rs cells rechallenged with Ras (Fig. 5D, inset). These data suggest that suppressed p53 activation allows rs cells to survive in the face of checkpoint activation and reduces apoptosis in response to rechallenge with Ras.

To confirm that Ras abrogated the response to DNA damage, we investigated the response of rs cells to the topoisomerase II inhibitor, etoposide. Etoposide induced G1 arrest in WRT cells (Fig. 6A), as reported for other normal cells (27). Remarkably, cell cycle progression in the rs cells (Fig. 6A) was unaffected by etoposide. Furthermore, the ability of rs cells to respond to etoposide with p53 phosphorylation or the induction of γH2AX was markedly attenuated (Fig. 6B). Etoposide stimulated apoptosis, as indicated by an increase in caspase-dependent (QVD-sensitive) hypodiploid DNA content, in parental cells (Fig. 6C). Apoptosis induced by etoposide was markedly suppressed in rs cells (Fig. 6C).
lysates were prepared and subjected to Western blotting for serine 15 phosphorylation of p53 and also conducted in rs 4 and rs 5 cells with similar results. Inhibitor QVD (50 μmol/L) blocked hypodiploid DNA content. The results shown are summarized from four independent experiments. Similar results were observed in rs 4 and rs 5 cells (data not shown). Inclusion of the pan-caspase inhibitor QVD (50 μmol/L) blocked hypodiploid DNA content, indicating that it reflects caspase-mediated cell death.

Collectively, these data support a novel mechanism for Ras transformation that entails the bypass of checkpoint-mediated cell cycle arrest and evasion of apoptosis, critical barriers that limit the expansion of damaged cells. Sustained down-regulation of the DNA damage response seems to mediate the induction of chromosomal instability by Ras.

Discussion

The analysis of Ras-transformed cells has provided substantial insight into the molecular basis of Ras transformation. Unfortunately, these models do not discriminate between the primary effects of Ras versus secondary changes that transpire following chronic exposure to Ras. To better model the contributions of Ras to tumor initiation, we investigated the acute effects of activated Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells. Thyroid cells were selected for analysis based on the frequent mutational activation of Ras in normal rat thyroid cells.

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The sustained induction of γH2AX in Ras-infected thyroid cells prompted us to investigate whether chromosomal instability was a consequence of cells bypassing the checkpoint in the presence of DNA damage. Despite the sustained induction of γH2AX, Ras-infected cells exhibited markers of mitotic cells, including serine 10 phosphorylation on histone H3 and MPM2 reactivity. Moreover, Ras dramatically increased the proportion of cells with micronuclei, an indicator of aberrant chromosome segregation and chromosome damage (24). These findings agree with a recent report that Ras induced bypass of G2 and spindle checkpoints in rat thyroid PC-Cl3.
cells (31). Interestingly, similar to the findings reported here, Ras also prolonged transit through S phase in PC-CL3 cells. Somewhat different from our data, the transit of Ras-expressing PC-CL3 cells through G2 phase was accelerated, and a significant proportion of these cells reentered G1 phase. This may indicate that sustained Ras activity is particularly incompatible with transit through early to mid-G1 phase, as Ras expression was induced at the G1-S boundary in the study by Knauf et al. (31). As reported here, inducible expression of activated Ras in PC-CL3 cells also induced chromosome fragmentation (32).

Given the high frequency of Ras mutations in human thyroid tumors, we assessed whether any of the cells that bypassed checkpoint activation were viable. Although the majority of Ras-infected cells perished via apoptosis, a subpopulation survived and could be propagated in the absence of overt selection for Ras. Remarkably, the surviving cells exhibited features of transformation, including alterations in cell morphology and growth regulation, and dedifferentiation. In that mutations that compromise checkpoint activation foster genetic instability, we were not surprised to find these cells exhibited profound chromosomal instability. However, the most striking feature of the surviving cells was the absence of γH2AX and suppression of p53 activation, despite the retention of a checkpoint response (Chk1 phosphorylation). When challenged with DNA-damaging agents, the Ras survivors failed to undergo cell cycle arrest or to induce γH2AX. These findings suggest that cells that survive exposure to Ras are impaired in their ability to respond to DNA damage. Perhaps as a consequence, the cells continue to proliferate in the face of chromosomal instability.

Studies done in γH2AX-deficient cells revealed that γH2AX is required for the detection of macroscopically visible repair foci, suggesting that it contributes to the reorganization of chromatin structure essential to DNA repair (20). More recently, it was shown that γH2AX prevents the progression of DNA double-strand breaks to chromosome breaks (33). This raises the intriguing possibility that the decrease in γH2AX in the Ras survivors contributes to the induction of chromosome instability. Suppressed activation of p53, a key mediator of cell cycle arrest upon DNA damage, would allow for the bypass of cell cycle checkpoints in the presence of DNA damage. Preliminary experiments failed to provide evidence for decreased levels of H2AX expression or enhanced dephosphorylation of γH2AX (34, 35) in the Ras survivors. This does not rule out the alteration of localized pools of chromatin-associated γH2AX by Ras. It is noteworthy that haploinsufficiency for H2AX induced chromosomal instability (36). Hence, diminished levels of properly localized γH2AX could result in an altered chromatin configuration that limits the access of repair proteins to sites of DNA damage. Overexpression of Myc in human fibroblasts disrupted DNA repair, resulting in a massive increase in chromosome breaks and translocations (37). The ability of Myc-expressing cells to repair lesions induced by direct scission of DNA was suppressed, indicating that the prolonged induction γH2AX observed in Myc-expressing cells was a consequence of defective repair, rather than the induction of oxidative DNA damage by Myc. γH2AX was rapidly induced and sustained in thyroid cells acutely exposed to Ras. Ras induction of γH2AX was insensitive to N-acetyl-cysteine, suggesting that DNA damage does not arise by oxidative damage. Rather, we speculate that Ras induces defects in DNA repair. In support of this notion, the Ras survivors were suppressed in their ability to induce γH2AX. Defects in the recognition or response to DNA damage, coupled with the bypass of cell cycle checkpoints, would provide a facile mechanism for the induction of chromosomal instability by Ras. The inability of surviving cells to initiate a DNA damage response would reinforce genome destabilization, and perhaps contribute to other indices of cell transformation. It is striking that γH2AX was diminished in high-grade lesions compared with hyperplasias (25), suggesting that a similar mechanism may be operative in humans.

In sum, our data support a novel mechanism for Ras transformation that entails the bypass of cell cycle checkpoints together with an impaired DNA damage response. Our results show that acute expression of activated Ras, in the absence of other signals, is sufficient to stimulate replication stress in thyroid cells with characteristics similar to those seen in precancerous lesions (25, 26). In addition to activating a checkpoint, Ras endows thyroid cells with the ability to bypass checkpoint activation in the face of DNA damage. Although this culminates in apoptosis in the majority of the cells, a subpopulation of cells evades apoptosis. These cells are endowed with altered growth properties that enable their expansion and emerge with hallmarks of transformation, including chromosomal instability. Hence, the induction of apoptosis by Ras in thyroid cells is not counterintuitive to the high frequency of Ras mutations observed in human thyroid tumors. Chromosomal instability is a hallmark of human tumors; however, how and when during tumor progression it arises remains unclear (38, 39). Our findings indicate that the Ras oncogene is capable of directly inducing chromosomal instability, an effect that may arise via cell cycle deregulation (40) together with defects in DNA repair. Considering that thyroid follicular carcinomas, tumors where Ras mutations are prevalent, are commonly aneuploid, it is intriguing to speculate that Ras endows these tumors with chromosomal instability.

References


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