Acquisition of Chemoresistance and Other Malignancy-related Features of Colorectal Cancer Cells Are Incremented by Ribosome-inactivating Stress*

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Colorectal cancer (CRC) as an environmental disease is largely influenced by accumulated epithelial stress from diverse environmental causes. We are exposed to ribosome-related insults, including ribosome-inactivating stress (RIS), from the environment, dietary factors, and medicines, but their physiological impacts on the chemotherapy of CRC are not yet understood. Here we revealed the effects of RIS on chemosensitivity and other malignancy-related properties of CRC cells. First, RIS led to bidirectional inhibition of p53-macrophage inhibitory cytokine 1 (MIC-1)-mediated death responses in response to anticancer drugs by either enhancing ATF3-linked antiapoptotic signaling or intrinsically inhibiting MIC-1 and p53 expression, regardless of ATF3. Second, RIS enhanced the epithelial-mesenchymal transition and biogenesis of cancer stem-like cells in an ATF3-dependent manner. These findings indicate that gastrointestinal exposure to RIS interferes with the efficacy of chemotherapeutics, mechanistically implying that ATF3-linked malignancy and chemoresistance can be novel therapeutic targets for the treatment of environmentally aggravated cancers.

Colorectal cancer (CRC)¹ is one of most commonly diagnosed cancers in developed countries, with a high morbidity and mortality rate because of metastasis via hepatic venous drainage from the original tumor mass (1, 2). This enables late-stage tumors to endow a very high metastatic potential to other organs, which is one of the important reasons for early treatment with surgical resection, chemotherapy, and radiotherapy for CRC patients. Although chemotherapy is the most general and effective strategy of treatment for metastatic colon cancer, occasional chemoresistance to anticancer drugs is a serious bottleneck for a successful cure. Fluorouracil (5-FU) and cisplatin are the first-choice chemotherapy drugs for metastatic colon cancer. 5-FU is a pyrimidine analog that causes DNA damage, ultimately inducing apoptosis. It is a representative chemical that induces tumor suppressor p53 (3, 4). p53 is a well-known tumor suppressor that is activated in response to oxidative stress, osmotic stress, and DNA damage and plays important roles in DNA repair, cell cycle arrest, and apoptosis (5, 6). As a crucial target of p53, regulation of macrophage inhibitory cytokine 1 (MIC-1) by nuclear translocated p53 or MIC-1-mediated activation of p53 as a positive feedback loop is an important apoptotic signaling pathway related to colon cancer treatment (7). Moreover, doxorubicin, a DNA-damaging agent, can also induce the p53 pathway. Also, an increase in expression of MIC-1 is observed during colon cancer treatment (8).

MIC-1, also known as growth differentiation factor 15 (GDF-15), non-steroidal anti-inflammatory drug-activated gene 1 (NAG-1), prostate-derived factor, placental bone morphogenetic protein, and placental TGF-β, belongs to the TGF-β superfamily, which has a critical role in regulating apoptosis and migration in colon cancer cells (9). When cancer cells undergo apoptosis in response to various anticancer agents, expression of MIC-1 increases, and cells expressing a low level of MIC-1 are less susceptible to apoptosis by external stimulation (9). In the case of various solid tumors, MIC-1 regulated by hormones including sex hormones (10) or retinoids (11, 12) induces caspase-dependent apoptosis by reducing antiapoptotic molecules. Given these, unveiling the regulatory mechanisms of p53 and MIC-1-mediated apoptotic signaling would be invaluable for understanding and improving cancer chemotherapy and related chemoresistance.

CRC has been widely considered an environmental disease, with a large proportion of cases from environmentally modifiable causes, including cultural, social, and lifestyle factors (13). We are exposed to various types of ribosome-related insults from the environment, dietary factors, and therapeutics (14–16). Among these, mucoactive ribosome-inactivating stress (RIS) as a potent etiological factor of epithelial inflammatory diseases has been investigated in various experimental models (17, 18). Specific ribosome-directed inactivating xenobiotics such as deoxynivalenol (DON) and anisomycin (ANS) damage the functionality of 28S ribosomal RNA during gene translation (19–23). This process results in the expression of genes important for cellular homeostasis as well as for a variety of processes involved in cell survival, proliferation, death, and inflammatory effects.
Ribosome-inactivating Stress-induced Chemoresistance

responses (21, 24). Among the stress-adapted genes, activation of transcription factor 3 (ATF3) plays an integral role in pathogenesis by external insults such as ischemic, respiratory and renal injury, carcinogens, mitogenic cytokines, or integrated stress, including RIS (25–27). ATF3 is a member of the mammalian ATF/cAMP responsive element-binding protein family of transcription factors, which has different functions depending on whether it is working as a homodimer or heterodimer with ATF2, c-JUN, JunB, or JunD (28, 29). Although ATF3 is involved in cellular apoptosis in some cases, it can also contribute to cell survival, regeneration, and proliferation in the context of detrimental injury, apoptotic signals, and embryonic development (16, 30). For instance, the level of ATF3 rapidly increases in response to tissue injury and is closely linked to survival and regenerative competence (31).

CRC as an environmental disease is largely influenced by accumulated epithelial stress from diverse environmental causes, including RIS. It was postulated that gastrointestinal exposure to RIS may influence the malignancy of epithelial tumor cells and their response to anticancer drugs. We revealed that ribosome-insulted CRC cells showed chemoresistance and changes in other chemoresponse-related malignancy properties, including epithelial-mesenchymal transition (EMT) and features of cancer stem-like cells (CSCs) in the ATF3-dependent pathway. These findings would improve our understanding of ribosomal perturbation in association with malignancy and chemotherapy, providing innovative strategies to enhance the efficacy of chemotherapeutics and tailored treatment of environmentally aggravated cancers.

Materials and Methods

Cell Culture and Reagents—HCT-116, a human colon cancer cell line, and the isogenic HCT-116 p53−/− cell line were provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD). A549, HepG2, A2780, HCT-8, DLD1, LOVO, SW480, LS174T, HCT15, and HT29 cells were purchased from the ATCC (Manassas, VA). Cells were maintained with RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 mg/ml streptomycin (all provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD), and the isogenic HCT-116 p53−/− cell line were provided by Dr. Jong-Sik Kim (Andong National University, Gyeongbuk, South Korea) and Dr. Seong-Joon Baek (University of Tennessee, Knoxville, TN). Cells were transfected with a shRNA vector using jetPRIME (polysulfate transfection) according to an allowed protocol. After transfection, cells were selected for 2 weeks with 400 mg/ml neomycin (Life Technologies). A selected single colony was expanded and maintained in RPMI 1640 medium supplemented with 10% FBS, 50 units/ml penicillin, and 50 mg/ml streptomycin with 200 mg/ml of neomycin.

Colony-forming Assay—10^5 HCT-8 cells were seeded in 100-mm plates with 500 ng/ml DON or 50 ng/ml of ANS for 24 h and treated with 50 ng/ml of F-U for 48 h. Forming colonies were cultured for an additional 12 days, exchanging medium every 3 days. Colonies were fixed with 100% methanol for 10 min and stained with 50% Giemsa solution. Colonies greater than 2 mm in diameter were counted, and the formation rate of the colony was calculated as colony number / number of cells seeded (10^5) × 100 (%) and statistically analyzed by one-way analysis of variance from four independent experiments.

Western Blotting Analysis—Protein expression was compared by immunoblot analysis using mouse monoclonal anti-human heterogeneous nuclear ribonucleoprotein, rabbit polyclonal anti-PARP1/2, mouse monoclonal anti-p53, and goat polyclonal anti-human GDF-15 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-rabbit secondary and anti-mouse secondary (Enzo Life Science, Plymouth Meeting, PA); and anti-goat secondary antibodies (Santa Cruz Biotechnology). Cells (8 × 10^5) were plated, maintained, and treated with chemicals. After treatment, detached and trypsinized cells were collected in a 15-ml conical tube. Collected cells were lysed in lysis buffer (1% (w/v) SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris (pH 7.4)) and sonicated for 30 s. Cell lysates containing proteins were quantified using WelProt (Welgene). 20-μg aliquots of proteins were separated by gel mini-electrophoresis (Bio-Rad). Proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ), and blots were blocked with 5% skim milk in TBST (Tris-buffered saline with 0.05% Tween 20) for 2 h and then probed with each antibody overnight at 4 °C. After washing three times with TBST, blots were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h and then washed with TBST for 10 min four times. Proteins were detected using an ECL substrate (ELPIS Biotech, Taean, South Korea).

Annexin V and Propidium Iodide (PI) Staining and Flow Cytometry Analysis—Cells (8 × 10^5) were seeded, maintained, and treated with chemicals. After treatment, detached and trypsinized cells were collected, washed with phosphate based saline (PBS) and suspended in 0.2 ml PBS. After adding 0.2 ml heat-inactivated and filtered FBS, the cells were fixed by slow dropwise addition of 1.2 ml ice-cold 70% (v/v) ethanol with gentle vortex and then incubated at 4 °C overnight. Fixed cells were centrifuged, washed with ice-cold PBS, and then incubated with 50 mg/ml RNase A at 37 °C for 30 min. Cells were resuspended with 1 ml propidium iodide (PI) DNA staining buffer (PBS containing 50 mg/ml PI, 50 mg/ml RNase A, 0.1 mM EDTA, and 0.1% Triton X-100). The cell cycle distribution for single cells was measured with a FACS Canto II (BD Biosciences) with cell aggregates. Apoptosis was assessed by measurement of PI-positive subdiploid DNA. For FITC-conjugated Annexin V and PI staining, har-
vested cells with trypsin were washed with PBS and resuspended with Annexin V-binding buffer. Cells were incubated with Annexin V (BD Biosciences, 1:40) and 100 μg/ml PI and analyzed by flow cytometry.

Immunofluorescence Analysis—Cells (1 × 10^6) were seeded in a glass-bottomed 35-mm cell culture plate. Then cells pre-exposed to 500 ng/ml DON or 50 ng/ml ANS for 24 h were washed with PBS and cultured with 10% FBS, RPMI for 3 days. Then cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS, permeabilized with 0.3% BSA and 0.2% Triton X-100 in PBS for 10 min, and blocked with 3% BSA in PBS for 2 h. Then cells were stained with DAPI (1:1000) and FITC-conjugated phalloidin antibody (1:1000). Nuclearily fragmented apoptotic cells were identified by DAPI staining and counted in more than six random fields for each sample.

Allograft of CMT-93—CMT-93, a C57BL/6 mouse colon cancer cell line, was purchased from the ATCC and maintained in DMEM (Welgene) supplemented with 10% (v/v) heat-inactivated FBS (Welgene), 50 units/ml penicillin, and 50 μg/ml streptomycin (Welgene) in a 5% CO_2 humidified incubator at 37 °C. CMT-93 pre-exposed to 500 ng/ml of DON for 24 h was dissociated into single cells with trypsin. 5 × 10^5 cells were resuspended with 200 μl of PBS and injected subcutaneously into the dorsal side of 14-week-old male C57BL/6 mice. Seven days later, 100 mg/ml 5-FU was intraperitoneally injected, and tumors were excised surgically 24 h later. The tumor volume was calculated and statistically analyzed by unpaired two-tailed t test.

Immunohistochemistry and Histological Analysis—Allograft tumors were assessed by immunohistochemistry according to a standard protocol with the following antibodies: MIC-1 (1:200, Santa Cruz Biotechnology), ATF3 (1:200, Santa Cruz Biotechnology), EGR-1 (1:200, Santa Cruz Biotechnology), E-cadherin (1:200, BD Biosciences), N-cadherin (1:200, BD Biosciences), and Vimentin (1:200, Cell Signaling Technology). 3,3′-diaminobenzidine-positive versus hematoxylin-positive cells were quantified by HistoQuest software (TissueGnostics) and statistically analyzed by unpaired two-tailed t test.

Spheroid Culture and Flow Cytometry—2.5 × 10^5 HCT-8 cells were seeded in an ultralow attachment 6-well plate (Costar) with RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin in a 5% CO_2 humidified incubator at 37 °C. Cells were pre-exposed to 500 ng/ml deoxynivalenol or 50 ng/ml anisomycin for 24 h, washed with RPMI 1640 medium three times, and then cultured for 6 days. Spheroid cells were dissociated into single cells by trypsinization, washed with PBS, and incubated with FITC-conjugated CD44 (BD Biosciences) and allophycocyanin (APC)-conjugated CD133 (MACS, Miltenyi Biotec) antibodies for 15 min, and then the expression of CD44 and CD133 positive cells was analyzed by flow cytometry (FACSCanto II, BD Biosciences).

Animal Ethics—This research was conducted in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Results

RIS induces Morphological Change and Resistance to Anticancer Drugs in Suspended Colon Cancer Cells—To assess the effects of environmental stress on circulating colon cancer cells detached from solid tumors, we simplified the methodology to mimic circulating tumor cells exposed to RIS under suspension conditions. Culture cells were pre-exposed to RIS before attachment to the culture plates and then stabilized to acquire a normal microenvironment to grow (Fig. 1A). First, we evaluated the effects of RIS on reattachment and survival of suspended colon cancer cells. Interestingly, HCT-116, a colorectal cancer cell line, pre-exposed to RIS by DON or ANS were weakly attached, not dead, round, and shrunken compared with control cancer cells with a polygonal shape with clear and sharp boundaries with neighboring cells (Fig. 1A, dashed box). Based on this experimental condition, the extent of RIS was titrated to the magnitude of translational inhibition by specific ribosome-directed inactivating xenobiotics (Fig. 1B). Next, in response to the 5-FU anticancer agent, numbers of detached and dead cells were significantly diminished by RIS pre-exposure in various types of cancer cells, including lung, liver, ovarian, and colorectal cancer cells. Interestingly, HCT-116 was the most sensitive to 5-FU compared with other cancer cell lines (Fig. 1C). Consistently, the cytotoxicity of 5-FU was also significantly reduced when cells were pre-exposed to RISs in HCT-116 colorectal cancer cells (Fig. 1D). Consistent with HCT-116, the cytotoxicity of 5-FU in various colorectal cancer cell lines such as LOVO, DLD-1, SW480, LS174T, HCT15, and HT29 was significantly decreased by RIS pre-exposure (Fig. 1E). Moreover, as the programmed cell death index, nuclear fragmentation (Fig. 1F), and numbers of Annexin V/PI-positive apoptotic cells (Fig. 1G) in response to 5-FU were also diminished by RIS pre-exposure. In addition to these acute anti-cytotoxic effects, RIS pre-exposure enhanced the 14-day colony survival response to 5-FU compared with 5-FU treatment alone (Fig. 1H). Taken together, these results indicate that RIS led to weak attachment of colon cancer cells with round morphology but attenuated chemosensitivity to anticancer drugs.

RIS-induced Chemoresistance to Anticancer Drugs Is Due to Attenuation of Proapoptotic Molecules—Drug resistance can be induced by various mechanisms, such as pumping out of drug, change of target molecule, interruption of drug influx, or increase in proliferation, including aberrant programmed cell death in response to anticancer drugs (32). In response to pro-apoptotic 5-FU, cleavage of poly(ADP-ribose) polymerase 1 (PARP-1), PARP1/2 and p53 induction was assessed as the representative pro-apoptosis readouts. 5-FU-induced increases in cytotoxicity and PARP-1 fragments were significantly reduced by RIS in dose-dependent manners (Figs. 2A and B). Moreover, p53 and its downstream target MIC-1, which mediates p53-dependent growth inhibition and apoptosis via regulation of p53 binding to its promoter (33), were also down-regulated by RIS pre-exposure (Fig. 2B). The alteration patterns of PARP-1 fragmentation in response to 5-FU and its inhibition by RIS were consistent with those of MIC-1 and p53 levels, suggesting involvement of p53 and MIC-1-mediated apoptosis in the mechanisms of chemoresistance to 5-FU. Of note, the dose-de-
A dependent decrease of cytotoxicity to 5-FU was significantly attenuated when p53 or MIC-1 was absent, confirming this rationale (Fig. 2A). Consistently, impairment of apoptosis in HCT-116 cells by RIS in response to 5-FU was consistent with that in p53−/− or MIC-1-deficient cells (Fig. 2C). In accordance with alteration of PARP-1 fragmentation, the 5-FU-dependent increase in sub-G1 apoptotic dead cells was also diminished in RIS-exposed and p53/MIC-1-deficient cells (Fig. 2D). Interestingly, p53 knockout cells did not express MIC-1 and vice versa, implying that recruitment of p53 and MIC-1 is responsible for 5-FU-induced apoptosis via positive feedback regulation. RIS significantly inhibited the expression of proapoptotic p53 and MIC-1 and perturbed 5-FU-induced apoptosis, resulting in the acquisition of chemoresistance to 5-FU. Flow cytometric analysis of apoptotic cells also confirmed this rationale. In contrast to the more than 50% increase in apoptosis in normal adherent cells in response to 5-FU, RIS-exposed or p53/MIC-1-deficient cells survived more than twice as long (Fig. 2D). RIS also induced resistance to another anticancer drug, paclitaxel, which is extensively used in the treatment of ovarian, breast, lung, and gastric cancers. Paclitaxel also led to cancer cell apoptosis via induction of MIC-1 and p53 proapoptotic molecules.
However, compared with the alteration pattern in response to 5-FU, enhancement of PARP-1 cleavage and the numbers of sub-G1 cells as readouts for apoptosis in response to paclitaxel were exclusively diminished in RIS-exposed or p53/MIC-1-deficient cells (Fig. 2E and F). Furthermore, paclitaxel-induced elevation of apoptosis was significantly inhibited by ANS, another RIS trigger, although the inhibitory effects of ANS were less than those of DON (Fig. 2G and H). Taken together, these results suggest that RIS could induce chemoresistance to anticancer drugs by inhibition of the positive feedback loop between MIC-1 and p53, which is responsible for RIS-induced apoptotic signaling.

RIS Can Induce Chemoresistance in Vivo—We showed that RIS could inhibit p53 and MIC-1-mediated apoptosis for anticancer drugs. The stress-induced regulatory mechanism in response to antitumor drugs is reminiscent of the acquisition of chemoresistance, resulting in resurgence and exacerbation of tumorigenesis during cancer treatment. To support this rationale, we performed an allograft tumorigenesis assay and examined whether RIS can attenuate the expression of proapoptotic...
signaling molecules in CMT-93, a mouse colorectal cancer cell line. CMT-93 cells pre-exposed to RIS by DON were subcutaneously injected into the dorsal region of C57BL/6 mice. Then 5-FU was additionally intraperitoneally injected, and allograft tumorigenesis was assessed in terms of tumor size and competence. RIS overtly resulted in an increase tumor size compared with the 5-FU-only control (Fig. 3A). Moreover, the expression of proapoptotic MIC-1 in the histological section of the allograft tumor was decreased by RIS (Fig. 3B), which was consistent with the in vitro cancer cells, as shown in Fig. 2. MIC-1 has a distinct binding site of the early growth response protein 1 (EGR-1) in its promoter and is transcriptionally enhanced by EGR-1-mediated tumor suppressor pathways (34, 35). In addition, ATF3-dependent attenuation of EGR-1 is important for the expression of MIC-1 and MIC-1-mediated apoptosis (16). Given this, we also measured the expression of MIC-1-associated transcription factors, including EGR-1 and ATF3, in the histological section of the allograft tumor. RIS significantly reduced the expression of EGR-1 and MIC-1 but enhanced that of ATF3, a negative transcriptional regulator of proapoptotic MIC-1 (Fig. 3B). These results indicate that RIS can worsen tumorigenesis via suppression of MIC-1-linked death signaling in response to the anticancer drug 5-FU.

**EGR-1, as a Crucial Target of ATF3, Is Required for Anticancer Drug-induced Apoptosis via MIC-1 Induction in Colon Cancer Cells**—We verified the involvement of EGR-1 as an initiating factor in p53- and MIC-1-dependent apoptosis in response to 5-FU. First, we confirmed that the level of EGR-1 was enhanced in response to 5-FU in a dose-dependent manner (Fig. 4A). Next, the effects of EGR-1 deficiency on apoptosis were assessed in 5-FU-treated cancer cells. Increased levels of p53, MIC-1, and PARP-1 cleavage in response to 5-FU were significantly reduced in EGR-1-deficient cells, like in RIS-exposed cancer cells (Fig. 4B). Consistently, PI-positive proapoptotic cells in response to 5-FU were also decreased in the EGR-1-deficient cells (Fig. 4C). We then tested whether EGR-1 overexpression can compensate for RIS-reduced proapoptotic signals. In contrast to our expectations, EGR-1 overexpression partly restored the levels of p53 and MIC-1, which were suppressed by RIS, although EGR-1 was definitely essential for the induction of proapoptotic molecules in response to 5-FU (Fig. 4D), implicating additional mechanisms of RIS-induced suppression of MIC-1 and p53 signals, such as translational arrest of MIC-1 and p53 by the intrinsic actions of RIS.

ATF3 is another representative stress-induced factor that was significantly increased in RIS-exposed allograft tumors.
Moreover, it is closely related to MAP kinase-mediated cell survival in RIS-linked cellular responses (36, 37). We thus tested whether RIS-induced ATF3 is dependent on the MAP kinase signaling pathway. RIS-induced ATF3 was significantly diminished by the inhibition of MAP kinases (Fig. 4E). Furthermore, anticancer drug-induced EGR-1 was repressed by ATF3 overexpression as well as RIS (Fig. 4F), implying an inverse relationship between ATF3 and EGR-1, as shown in the RIS-exposed allograft tumor model (Fig. 3). These results raised the possibility that RIS-induced ATF3 can decrease EGR-1-promoted MIC-1 expression and subsequent apoptosis in response to anticancer agents. As predicted, ATF3 deficiency restored 5-FU-induced EGR-1 expression, which was attenuated by RIS (Fig. 4G). However, MIC-1 expression and subsequent apoptotic signaling (PARP1/2 cleavage) were only partly compensated by ATF3 deficiency (Fig. 4G). This discrepancy indicates that the globally inhibitory intrinsic actions of RIS on ribosome and translational arrest of proapoptotic MIC-1 expression incapacitated the compensatory action on MIC-1 expression by ATF3 suppression or EGR-1 overexpression in RIS-exposed cancer cells (Fig. 4F and G, respectively). Taken together, anticancer drug-induced EGR-1 promotes MIC-1-mediated apoptosis in cancer cells, whereas it was negatively regulated by RIS-induced ATF3. However, simple genetic suppression of ATF3 was not enough to enhance cancer cell apoptosis in response to anticancer drugs. The protein synthesis of proapoptotic molecules such as MIC-1 and p53 is blocked by intrinsic action of RIS, which failed to increase chemosensitivity in CRC cells.

Ribosome-inactivating Stress-induced Chemoresistance

MAY 6, 2016 • VOLUME 291 • NUMBER 19
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Ribosome-inactivating Stress-induced Chemoresistance

FIGURE 4. RIS exposure causes ATF3-dependent suppression of EGR-1, an apoptosis-triggering factor. A, HCT-116 cells cultured with 10% FBS and 1% P/S RPMI for 48 h were exchanged with FBS-free RPMI for 12 h, treated with 100 μM 5-FU, harvested at the corresponding time points, and evaluated for the level of EGR-1 by Western blotting. hnRNP, heterogeneous nuclear ribonucleoprotein. B and C, HCT-116 cells pre-exposed to 500 ng/ml DON for 24 h and EGR-1 stable knockdown cells by shRNA against EGR-1 were examined for proapoptotic signaling molecules in response to 100 μM 5-FU for 48 h by Western blotting (B) and PI-positive flow cytometric quantification (C). D, empty vector- or EGR-1 expression plasmid-transfected HCT-116 cells were pre-exposed to 500 ng/ml DON for 24 h and assessed for levels of PARP1/2 fragmentation, p53, and MIC-1 in response to 100 μM 5-FU for 48 h by Western blotting. E, levels of ATF3 were analyzed in the HCT-116 cells pre-exposed to 500 ng/ml DON along with 5 nM SB203580 (SB, a MAP kinase inhibitor), 5 nM SP600125 (SP, a JNK inhibitor), and 1 nM U0126, (an ERK inhibitor) for 2 h. Dotted box, inhibitory effects of SP600125 or U0126 on JNK and ERK activation in response to treatment with DON and ANS for 1 h were evaluated by Western blotting in HCT-116 cells. Con, control; Veh, vehicle; F, HCT-116 cells pre-exposed to 500 ng/ml DON for 24 h or ATF3-overexpressing cells were evaluated for levels of EGR-1 in response to 5-FU. G, control or ATF3 antisense (AS)-expressing cells pre-exposed to 500 ng/ml DON for 24 h were treated with 100 μM 5-FU for 48 h and analyzed with proapoptotic signaling molecules.
tasis, is a following adaptive change during chemotherapy, and mesenchymal-like cancers are susceptible to exacerbating resistance to anticancer drugs (38, 39). In the line of this, we analyzed the alteration of E-cadherin, N-cadherin, and Vimentin in allograft tumor tissues originated from RIS-exposed colon cancer cells. Expression of E-cadherin, an epithelial cell marker, was decreased; however, mesenchymal markers, including N-cadherin and Vimentin, were enhanced by RIS (Fig. 5A). As an additional factor of cancer cell differentiation mediating RIS-induced chemoresistance to anticancer drugs through progression of EMT, the biogenesis of CSCs was also observed. We assessed the alteration of CSCs in anchorage-independent spheroid cultures. CD44 and CD133 are surface proteins that are extensively used to evaluate CSCs, especially colonospheres (40, 41). When colorectal cancer cells were exposed to RISs, CD44- and/or CD133-positive CSC populations were significantly increased (Fig. 5B). As shown in Fig. 5A, chronic exposure of tumor cells to RIS can lead to bidirectional inhibition of cell death in response to anticancer drugs, resulting in perturbation of effective chemotherapy. First, RIS attenuated MIC-1- and p53-dependent apoptotic signaling via inhibition of EGR-1 by MAP kinase-dependent ATF3. Second, RIS intrinsically led to translational arrest of MIC-1 or p53 expression regardless of ATF3. In any regulatory pathway of MIC-1- or p53-mediated cancer cell apoptosis, RIS hindered the proapoptotic process and enhanced the chance of cancer cell survival from chemotherapy.

Transformation of chronically insulted enterocytes to malignant phenotypes requires EMT, which enhances the anchorage-independent survival potential of circulating cancer cells to perform the metastasis process of CRCs (14). Malignant epithelial cells with metastatic potential are resistant to anoikis, a form of apoptosis induced by anchorage-dependent cells upon loss of attachment from the extracellular matrix, and able to

Discussion

We are exposed to diverse types of environmental RIS, including UV irradiation, ribosome-inactivating food toxicants, and drugs, including anisomycin, trichothecene mycotoxins, ricin, and shiga-like toxins. In this study, RIS triggered chemoresistance to anticancer drugs via attenuating MIC-1-mediated proapoptotic signaling. As shown in Fig. 6, chronic exposure of tumor cells to RIS can lead to bidirectional inhibition of cell death in response to anticancer drugs, resulting in perturbation of effective chemotherapy. First, RIS attenuated MIC-1- and p53-dependent apoptotic signaling via inhibition of EGR-1 by MAP kinase-dependent ATF3. Second, RIS intrinsically led to translational arrest of MIC-1 or p53 expression regardless of ATF3. In any regulatory pathway of MIC-1- or p53-mediated cancer cell apoptosis, RIS hindered the proapoptotic process and enhanced the chance of cancer cell survival from chemotherapy.

Transformation of chronically insulted enterocytes to malignant phenotypes requires EMT, which enhances the anchorage-independent survival potential of circulating cancer cells to perform the metastasis process of CRCs (14). Malignant epithelial cells with metastatic potential are resistant to anoikis, a form of apoptosis induced by anchorage-dependent cells upon loss of attachment from the extracellular matrix, and able to
survive in the circulation after detachment from their extracellular matrix and being endowed with metastatic properties. It has been demonstrated that ANS can sensitize cancer cells to anoikis via insulting antiapoptosis pathways (42). In contrast, this study demonstrated that pre-exposure to RIS, including ANS, triggered ATF3-mediated cell survival signaling, which attenuated MIC-1-activated proapoptotic responses in cancer cells under chemotherapeutic stress. Although acute high doses of ANS can be adverse to cell proliferation or cytotoxic, extended exposure to RIS triggers cytoprotective ATF3 production, which counteracts RIS-induced cytotoxicity (16). ATF3 can protect cells from physical and chemical cytotoxic stresses by suppressing the induction of cell death factors such as p53 proteins (43). In response to injuries, ATF3 up-regulates a survival factor, heat shock protein HSP27, as well as c-Jun/Fos protein, without which cells will undergo a retarded regeneration after injury (44). Moreover, ATF3 can also facilitate malignant tumor metastasis because EMT-related genes, including TWIST1, FN-1, Snail, and Slug, were also up-regulated by ATF3 (45). In particular, ATF3 gene copy numbers and protein levels are significantly higher in malignant breast tumors (46). Moreover, because ATF3 can be up-regulated by diverse xenobiotics with anticancer activities such as non-steroidal anti-inflammatory drugs, anti-inflammatory polyphenol curcumin, and green tea catechins, the pro-survival and EMT-triggering activities of ATF3 can have potentially adverse effects on anticancer chemotherapy.

In addition to the effects of RIS on cancer cell viability, this study suggested evidence that RIS can alter the biogenesis of CSCs. A strong association between EMT-linked malignant properties and CSCs has been demonstrated, and this association can reinforce cancer cells to be more resistant to anticancer drugs (47). For instance, the CD44+/CD24− expression pattern associated with CRCs had higher EMT gene expression patterns than more differentiated tumor cells (48). As more evidence, forced expression of snail in CRC cell lines elevates chemoresistance and CSC phenotypes, including enhanced spheroid formation as well as increased CD133 and CD44 expression (49). Therefore, targeting CRC stem-like cells in combination with conventional therapies would be a promising approach for overcoming tumor recurrence and metastasis because CSCs are resistant to standard cancer therapies, such as radiation and chemotherapy. Therefore, it can be speculated that RIS-induced EMT can be positively associated with the increment in colon CSCs in this study. Moreover, ATF3 was positively involved in the increase in the cancer stem-like population by RIS (Fig. 5C). Because ATF3 enhances EMT phenotype-related genes (45), altered EMT may influence the CSC phenotype and resistance to chemotherapy. There is evidence that ATF3 enhances cancer-initiating cell features, as indicated by its ability to increase the population of CD24low−CD44+ high cells, mammosphere-forming ability, and tumor-initiating frequency in breast cancer (45). Particularly stromal stressors, including pro-inflammatory mediators, endoplasmic reticulum
Ribosome-inactivating Stress-induced Chemoresistance

stress, and hypoxia in the tumor microenvironment, occasionally induce expression of ATF3, an adaptive-responsive gene (45, 50). Therefore, ATF3, as a hub of the procarcinogenic biological network, promotes malignancy by increasing the metastasis and biogenesis of CSC features. Although the detailed molecular mechanisms of biogenesis of the CSC population via ATF3 need to be addressed, our finding fortifies the assumption that intervention with ATF3 can be clinically relevant in cancer chemotherapy. In this study, RIS also induced ATF3 as an adaptive-responsive gene that retarded MIC-1- or p53-mediated cancer cell death and consequently hindered the actions of chemotherapy. However, simple suppression of ATF3 was not enough to increase the chemosensitivity in this model (Fig. 4) because RIS intrinsically blocks the protein biosynthesis of proapoptotic genes regardless of ATF3 action. Therefore, RIS in the tumor niche makes chemotherapy more complicated, and additional strategies, including regulation of ribosomal integrity as well as ATF3 suppression, should be developed to enhance the efficacy of chemotherapy.

In conclusion, gastrointestinal exposure to RIS interferes with the efficacy of chemotherapeutic agents, mechanistically implying that ATF3-linked malignancy and chemoresistance can be novel therapeutic targets for the treatment of environmentally aggravated cancers. Another insight from the mechanism study is that RIS-pre-exposed hosts pose more risk of increased chemoresistance to anticancer agents when they need to get chemotherapy against cancers, which needs to be further investigated in the clinical field.

Author Contributions—C. K. O., S. J. L., and Y. M. conceived the research projects and hypotheses. C. O. conducted most experiments, and S. J. L. conducted the experiments related to EMT and cancer stem cells. S. H. P. contributed to animal exposure. S. J. L., C. K. O., and Y. M. wrote the paper. Y. M. supervised the overall project.

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