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Reactive Oxygen Species Induced By the Compound Bisphenol-a-Diglycidyl-Ether Cause Senescence and Apoptosis in Colorectal Cancer Cell Lines Regardless of MDR1 or p53 Status

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Background: Reactive oxygen species (ROS) can be toxic. Tumors have elevated ROS, and the ability of tumor cells to accommodate additional ROS may be limited compared to normal cells, suggesting the possibility of "redox therapy". ROS are critical to the mechanism of many cancer drugs, and experimental agents that affect ROS metabolism are being tested. However, this strategy has been difficult in colorectal cancer (CRC) due to high level expression of drug efflux pumps and loss of the stress-activated p53 pathway that normally controls expression of ROS scavenging enzymes. We show that ROS induced by the compound bisphenol-A-diglycidyl-ether (BADGE) cause senescence and apoptosis in CRC cell lines, including those with high levels of MDR1 efflux pump activity and loss of p53 function. Methods: ROS, Jnk kinase signaling, apoptosis, and senescence (irreversible cell cycle arrest) were measured in HT-29, SW620, HCT-116, and HCT-15 cells. ROS were measured using the redox-sensitive dye DCFDA. Jnk kinase activation was measured by Western blot, and apoptosis by annexinV staining. Markers of senescence included large cells with vacuolated cytoplasm, ability to maintain constant viable cell numbers over three weeks, and appearance of senescence-associated beta-galactosidase. Results: BADGE treatment caused immediate (<1h) dose-dependent increases in ROS that lasted for at least 12h. At later times, a further increase in ROS corresponding to apoptosis was observed. The consequences of BADGE treatment were proportional to the amount of ROS generated. 50μM BADGE caused senescence but not apoptosis, while higher doses (75-100μM) triggered ROS and Jnk kinase-dependent apoptosis. Increasing glutathione-dependent ROS scavenging with N-acetylcysteine allowed greater doses of BADGE to be tolerated, while reducing glutathione with buthionine sulfoximine sensitized the cells to BADGE. BADGE triggered sustained Jnk kinase activation, and Jnk inhibitors decreased the pro-apoptotic effects of BADGE. The concentration of BADGE resulting in half-maximal apoptosis (IC50) was 75μM in all cell lines, regardless of MDR1 or p53 status. The MDR1 inhibitor verapamil, while reducing efflux of rhodamine 123, did not increase the IC50 of BADGE in HCT-15 cells. BADGE concentrations that caused ROS and apoptosis did not suppress PPARγ-dependent transcription, and PPARγ knockdown did not replicate or interfere with the effects of BADGE, suggesting that the effects of BADGE are PPARγ-independent. Conclusions: Depending on dose, ROS lead to senescence or apoptosis even in MDR1 high p53 mutant CRC cells. BADGE is a possible candidate for CRC redox therapy.

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Novel p53-Derived Peptide Induces Extensive Necrosis in Cancer Cells

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INTRODUCTION: The p53 protein is known to counteract oncogenic effects of ras-p21. Therefore, molecules and pathways associated with p53 are critical targets for the development of anti-cancer therapeutic agents. Utilizing a computer-based molecular modeling approach, anti-cancer peptides were designed from p53 that potentially block the proliferation of cancer cells. METHODS: The synthesized peptide corresponds to the hdm-2-binding domain (12-26) of p53 linked to a trans-membrane penetrating sequence. We examined whether this construct (PNC-27) induced anti-cancer activity in several ras-transformed human cancer cell lines: pancreatic cancer (MiaPaCa-2), breast cancer (MCF-7), and sarcoma (HT-1080). Control groups received an unrelated PNC- peptide. All cell lines were treated daily with PNC-27 peptide. During this time, we recorded changes in cell morphology and growth characteristics among treated and control groups. Peptide mediated necrosis was determined by measuring lactate dehydrogenase (LDH) as well as elevation of apoptotic proteins (caspase 3, 7) to elucidate the anticancer mechanism. RESULTS: Tumor cell death was initiated at 20ug/ul of PNC-27, with total cancer cell killing observed at 160ug/ul after 3 days of treatment. Cancer cell death via necrosis was demonstrated by increased levels of LDH release within 4 hours of PNC-27 peptide treatment for all ras-transformed human cancer cell lines. These findings were not seen in control groups treated with the unrelated PNC-peptide. In contrast, during peptide treatment there was no elevation of apoptotic proteins (caspase 3, 7) detected. Morphologically, treated cancer cells were noted to form aggregates then cellular clumps. These observations became more prominent as peptide dose increased during the course of treatment. CONCLUSIONS: These findings indicate that the mechanism of cell death with this anti-cancer peptide is via necrosis, not apoptosis. Remarkably, it appears that this novel p53-derived peptide has anti-cancer activity against a spectrum of human cancer cell lines. These results suggest that the therapeutic effects of PNC-27 may also be applicable to many types of human gastrointestinal malignancies.

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Suppression of Prohb-EGF Carboxy Terminal Fragment Nuclear Translocation Might Be a New Molecular Targeting Therapy for Gastric Cancer

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Background: Recently, it is remarkable that molecular target therapy has been developed, and it has been one of the effective therapies for various cancers. Especially, epidermal growth facter receptor (EGFR) is one of the most attractive target for cancer therapy and many studies about EGFR were reported in experimental models of epitherial cancers. Anti-EGFR therapies such as cetuximab and erlotinib generally have been used for several cancers, but no molecular target agent with clinically sufficient effects on gastric cancer has yet been approved. On the other hand, little attention has been paid to remnant cell-associated domains created by cleavage of EGFR ligands. A recent study using human primary keratinocytes showed that cleavage of membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF) induced translocation of the carboxy terminal fragment of proHB-EGF (HB-EGF-CTF) from the plasma membrane to the nucleus and regulated cell cycle. We speculated that not only the inhibition of EGFR phosphorylation but also the inhibition of HB-EGF-CTF nuclear translocation is crucial to inhibit cancer cell proliferation. Thus we studied the effect of suppressing HB-EGF-CTF nuclear translocation in gastric cancer cell lines. Methods: Two gastric cancer cell lines, MKN28 and NUGC4, were used. KB-R7785, an inhibitor of proHB-EGF shedding, was used to suppress HB-EGF-CTF nuclear translocation with cetuximab which inhibits EGFR phosphorylation. Cell growth was analyzed by MTS assay, apoptosis was evaluated by the assay of caspase-3 and -7 and cell cycle was investigated by flow cytometry. Results: 12-O-tetradecanoylphorbol-13-acetate (TPA) induced proHB-EGF shedding and accelerated nuclear translocation of HB-EGF-CTF. Imunofluorescence study confirmed that KB-R7785 suppressed HB-EGF-CTF nuclear translocation by inhibiting the shedding of proHB-EGF. KB-R7785 inhibited cell growth in a dose-dependent manner ($p<0.01$). KB-R7785 also induced G1-phase accumulation, but barely induced apoptosis except in NUGC4 cells with high-dose KB-R7785. KB-R7785 suppressed cyclin A and c-Myc expression that are the targets molecules activated by HB-EGF-CTF nuclear translocation. Conclusions: Not only the inhibition of EGFR phosophorylation but also the inhibition of HB-EGF-CTF nuclear translocation plays crucial roles in inhibitory regulation of cancer cell growth. Suppression of HB-EGF-CTF nuclear translocation might be effective as a new molecular targeting therapy for gastric cancer.

W1963

Sumoylation Enhances Nuclear Localization of KrüPpel-Like Factor 5

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BACKGROUND and AIM: Krüppel-like factor 5 (KLF5) is a transcription factor abundantly present in the crypt epithelial cells of the intestine. Previous studies demonstrated that KLF5 is important in regulating several physiological processes including proliferation of intestinal epithelial cells. KLF5 is primarily localized to the nucleus but little is known about the mechanism that regulates its nuclear targeting. One process that influences cellular distribution of transcription factors is SUMOylation, a covalent modification of lysine residues with small ubiquitin-like modifier (SUMO). Here we aim to determine whether KLF5 is post-translationally modified by SUMO and whether SUMOylation regulates its nuclear localization. **METHODS**: SUMOylation of KLF5 was determined by immunoprecipitation and Western blotting of cells transfected with HA-tagged KLF5 and GFP-tagged SUMO-1. Site-directed mutagenesis of KLF5 was conducted to determine which lysine residues are SUMOylated. Immuno- or GFP-fluorescence microscopy was conducted to determine the subcellular distribution of wild type, mutant or fusion KLF5 constructs in transfected cells. **RESULTS**: SUMOylation of KLF5 was demonstrated by transfection of COS-1 cells with HA-KLF5 and GFP-SUMO-1 and subsequent immunoprecipitation with HA antibody, followed by Western blotting for HA and GFP. This modification was also apparent when whole cell lysates were analyzed by Western blotting for HA. In addition, SUMOylation of endogenous KLF5 was detected by Western blotting for KLF5. Site-directed mutagenesis identified two specific lysine residues within KLF5 as the targets of SUMOylation that reside in motifs resembling a consensus SUMOylation site. Compared to wild type KLF5, KLF5 SUMOylation mutants showed decreased localization to the nucleus, with a concomitant increase in cytoplasmic localization. We noted that immediately adjacent to the first SUMOylation motif of KLF5 is a leucine-rich sequence (LRS) that resembles a consensus nuclear export signal (NES). Fusion of this LRS to GFP resulted in a significant redistribution of the normally diffuse GFP to the cytoplasm. This cytoplasmic targeting of GFP by LRS was inhibited by the nuclear export inhibitor, leptomycin, indicting that LRS directs nuclear export. Importantly, the addition of the first SUMOylation motif in KLF5 to the GFP-LRS fusion protein also reduced its cytoplasmic targeting, suggesting that SUMOylation inhibits nuclear export. **CONCLUSIONS**: Results of this study demonstrate that KLF5 is post-translationally modified by SUMOylation and that this modification enhances KLF5's nuclear localization by inhibiting a novel nuclear export motif within KLF5.

W1964

Telomerase Reverse Transcriptase Regulation By TGF-β Signaling Through Adaptor ELF and SMAD3 That Is Independent of C-MYC

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TGF-β induces antiproliferative responses in many cancer cells by suppression G1/S cell cycle regulators c-Myc and cyclin-dependent kinases (cdks). Myc amplification, telomere maintenance, and telomerase reverse transcriptase (TERT) reactivation are common features of human foregut cancers, such as hepatocellular carcinoma (HCC). Emerging evidence indicates that ELF, a Smad3/Smad4 adaptor protein required for TGF-β signaling, is a powerful tumor suppressor [Science, 2005, 310(5745):68-71, Oncogene, 2007, 26(50):7103-10]. *Elf*$^{+/-}$, *elf*$^{+/-}$*/Smad3*$^{+/-}$ and *elf*$^{+/-}$*/Smad4*$^{+/-}$, (but not *Smad3*$^{-/-}$) mice dramatically develop foregut cancers, including hepatocellular, pancreatic and gastric cancers. However, the specific role(s) of h-TERT, c-MYC and ELF, and their relation to the TGF-β pathway, in foregut cancer formation are poorly understood. Deletion of ELF results in a dramatic and spontaneous formation of liver and gastrointestinal cancers, with exon 15 mutations in 11% of human HCC and gastric cancer cell lines tested. Aims: In this study, we investigated the mechanism of role of Elf, Smad3 and c-Myc in regulating human TERT gene expression by TGF-β in HepG2, PLC/PRF/5, SNU 298 cell lines and *elf*$^{+/-}$*/Smad3*$^{+/-}$ mice tumor tissues *In Vitro* and *In Vivo*. Results show that: 1) The *Elf*$^{+/-}$*/Smad3*$^{+/-}$mice develop visceromegaly and multiple cancers, including metastatic pancreatic, hepatocellular, small bowel lymphomas, adrenocortical carcinomas, renal carcinomas and tumor tissues from *elf*$^{+/-}$*/Smad3*$^{+/-}$ mice show a dramatic decrease of ELF mRNA; 2) TERT and c-Myc are markedly elevated in *elf*$^{+/-}$ and *elf*$^{+/-}$*/Smad3*$^{+/-}$ mice; Interestingly, TERT levels are far higher than can be accounted for by c-Myc levels in *elf*$^{+/-}$ and *elf*$^{+/-}$*/Smad3*$^{+/-}$ tumors; 3)Ectopic ELF and Smad3 suppress TERT greater than c-Myc in the absence of TGF-β. 4) Both ELF and Smad3 associate with c-Myc in TGF-β stimulated hepatocytes and suppress TERT. 5) Overexpression of ELF and/or Smad3 decreases hTERT RNA levels in PLC/PRF/5 and SNU 298 human cell lines. Conclusions: Taken together our preliminary data suggest that divergent pathways converge on ELF and Smad3 that then regulate TERT. Inactivation of the TGF-β signaling pathway with