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Original Article

Silencing of the mRNA-binding protein HuR increases the sensitivity of colorectal cancer cells to ionizing radiation through upregulation of caspase-2

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ABSTRACT

Increased abundance of the mRNA-binding protein human antigen R (HuR) is a characteristic feature of many cancers and frequently associated with a high grade malignancy and therapy resistance. HuR elicits a broad cell survival program mainly by stabilizing or increasing the translation of mRNAs coding for anti-apoptotic effector proteins. Conversally, we previously identified the pro-apoptotic caspase-2 as a novel HuR target which is mainly regulated at the level of translation. In this study, we investigated whether siRNA-mediated HuR knockdown interferes with cell survival and radiation sensitivity by monitoring apoptosis, DNA repair and three-dimensional (3D) clonogenic survival. We observed a significant elevation in caspase-2 upon HuR depletion and in turn, a sensitization of colorectal DLD-1 and HCT-15 cells to radiation-induced apoptosis as implicated by the dose-dependent elevation of sub-G_1 phase cell entry and increased caspase-2, -3 and poly ADP-ribose polymerase (PARP)-cleavage, respectively. Coincidentally, HuR deficiency significantly elevated the number of radiation-induced γH2AX/53BP1-positive foci indicating an increase in DNA damage. Accordingly, the irradiation-dependent reduction in clonogenic cell survival was further impaired after knockdown of HuR. Importantly, HuR knockdown remained ineffective to radiation-induced cell responses after additional knockdown of caspase-2. Furthermore, by using RNA-pull down assay we demonstrate that irradiation (6 Gy) robustly increased HuR binding to caspase-2 mRNA. Collectively, sensitization of colon carcinoma cells to radiation-induced cell death and DNA-damage by HuR knockdown critically depends on caspase-2 and may represent a valuable approach to intervene with therapy resistance of colorectal cancer (CRC).

Introduction

Colorectal cancer (CRC) is one of the most common cancers in the western world. Despite current treatment options including surgery, chemo- and radiotherapy, these therapies are still associated with numerous side effects and with the occurrence of therapy resistance. For this reason, an implementation of molecular approaches aiming on a sensitization of CRC cells is a strong need to improve the efficacy of current tumor therapies. Experimental evidence of the last decade implicates that besides variations in the transcriptome of tumor cells, post-transcriptional mechanisms play an important role for an increased cell survival of tumor cells [for review see: Refs. [1,2]]. In line with that, the RNA-binding protein HuR which regulates AU- and U-rich element (ARE) bearing mRNAs, among many of them encoding cancer-related proteins, has emerged as a promising target of potential cancer therapeutics. Upon binding to these RNA signatures which in most cases reside within the 3′untranslated region (3′UTR) of the mRNA, HuR can affect many fates of an mRNA including splicing, stabilization, intracellular transport and translation [for a review see: Refs. [3,4]]. The critical impact of HuR-dependent posttranscriptional gene regulation in carcinogenesis is highlighted in many tumors wherein

Abbreviations: CRC, colorectal cancer; DDR, DNA-damage response; HuR, human antigen R; PARP, poly ADP-ribose polymerase; 5′UTR, 5′-untranslated region.

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the increased HuR abundance tightly correlates with high-grade malignancy and with a poor outcome as has been extensively documented in human colon cancer [5–7]. Prominent target genes of HuR implement key regulators of cell cycle control, angiogenesis, cancer cell invasion or metastasis and evasion of immune recognition [3,4]. In addition, HuR elicits a broad cell survival program mainly by stabilizing and/or enhancing the translation of prominent anti-apoptotic effector proteins [1,4].

Since some prominent HuR target genes including the X-linked inhibitor of apoptosis (XIAP) and Survivin are also involved in radiation resistance [8,9], the anti-apoptotic program by HuR becomes particularly relevant under conditions when tumor cells are exposed to ionizing radiation. Mechanistically, radiation of cells initiates the DNA damage response (DDR), a network of signaling pathways which coordinate cell cycle checkpoints and arrest, DNA repair or apoptosis [10–12]. Increasing evidence from many laboratories indicates that evasion from DDR-induced cell death predisposes cells to malignancy and furthermore induces an important step towards therapy resistance of tumor cells [13,14]. Importantly, signaling pathways of the DDR can jointly influence HuR activity either by the alteration of cytoplasmic HuR abundance and/or by affecting HuR's binding affinity to target mRNAs [15–17]. Searching for genes functionally relevant for apoptosis resistance in colorectal carcinoma cells, we previously have identified the pro-apoptotic Caspase-2 (synonym: *Caspase-2L*, *ICH-1L*) as a novel target of HuR [18]. In contrast to most prototypical target genes, HuR inhibits caspase-2 translation via a constitutive binding to the 5′untranslated region (UTR) thereby conferring a reduced sensitivity towards drug-induced apoptosis [18]. Unlike other caspases, the defined role of caspase-2, the evolutionally most conserved caspase in apoptotic pathways is not fully understood. However, caspase-2 by acting as an apical caspase is critically involved in the intrinsic, mitochondrial apoptotic pathway induced by genotoxic stress, chemotherapeutic drugs and ionizing radiation [19–21]. Functionally, a deficiency of caspase-2 promotes an aberrant DDR and genetic instability [22,23].

Given the functional impact of HuR for tumor cell survival, we investigated whether siRNA-mediated depletion of HuR would sensitize colorectal cancer cells to ionizing radiation-induced cell death and consequently may impair clonogenic survival in three-dimensional (3D) grown colorectal cancer cells. The functional contribution of caspase-2 in the HuR depletion-dependent sensitization to irradiation-induced apoptosis and clonogenic survival was tested by double knockdown of HuR and caspase-2.

Materials and methods

Reagents

All cell culture media, supplements and modifying enzymes were purchased from Invitrogen (Karlsruhe, Germany). Laminin-rich extracellular matrix (lrECM; BME Growth Factor Reduced PathClear) was obtained from Biozol (Eching, Germany).

Cell culture

The human colorectal carcinoma cell lines DLD-1 and HCT-15 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and from the American Type Culture Collection (LGC-Promochem, Wiesbaden, Germany), respectively. Both cell lines are heterozygous for p53 mutation [24]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO_2 and 95% humidity.

RNA interference

Transfection of subconfluent cells with siRNAs was performed by using the Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Gene silencing was performed by transfecting 50 nM of a mixture of small interfering RNA (siRNA)-duplexes from Santa Cruz (Heidelberg, Germany; siRNA-HuR, sc-35619) and FlexiTube siRNAs for human HuR (SI00300139, SI03166436, SI103246551 and SI03246887) or the same amount of siRNAs for caspase-2 (SI00299551) from Qiagen (Hilden, Germany). A non-targeting control siRNA (D001206-13) was from Dharmacon (Fermentas, St. Leon-Rot, Germany). For double siRNA transfections, each siRNA was applied at 25 nM. 48 h after transfection, the cells were treated for specific applications before lyzed for Western blot anaylsis.

Irradiation procedure

Irradiation with single doses of 2, 4 or 6 Gy was performed using a linear accelerator (SL-15, Elekta, Crawley, UK) with 6 MeV/100 cm focus-surface distance and a dose rate of 4 Gy/min. To guarantee equal conditions untreated cells were kept under equivalent surroundings in the irradiation control room.

Western blot analysis

Whole-cell lysates were prepared as described previously (Winkler et al., 2014). Total cell lysates containing 50 μg of protein were prepared in SDS sample buffer and resolved by 12%–15% SDS-PAGE, and transferred for immunodetection with the following specific primary antibodies: anti-HuR (sc-5261) and anti-BID (sc-373939), both from Santa Cruz, anti-caspase-2 (#611022, BD Biosciences, Heidelberg, Germany), anti-caspase-3 (#9662) and anti-PARP (#9542) both from Cell Signaling, (Frankfurt, Germany). For detection, blots were incubated with goat anti-rabbit (sc-2054) or goat anti-mouse (sc-20559) HRP-linked antibodies (Santa Cruz Biotechnology) and finally visualized with chemiluminescence using an ECL system from Amersham Biosciences (Freiburg, Germany). To confirm equal loading of protein amounts, blots were re-probed with a β-actin antibody (#A2228, Sigma–Aldrich Deisenhofen, Germany).

Biotin pull-down assay

Biotin pull-down assay was performed as described previously [18]. Briefly, a biotinylated RNA sense probe was generated by using 10 μg of linearized plasmid pCR2.1-5′-UTR-caspase-2 using the "RiboMax Large scale RNA production system T7" (Promega, Mannheim, Germany) and T7 RNA polymerase and biotin-CTP (Invitrogen). 15 μg of the biotinylated RNAs were conjugated to streptavidin-conjugated agarose beads in incubation buffer (10 mM Tris–HCl, pH 7.5; 150 mM KCl; 1.5 mM MgCl2; 0.5 mM DTT; 40 U/ml RNasin) at 4 °C for 2 h with continuous rotation. Subsequently, 300 μg of total cell lysates were added to the beads and incubated for 45 min at 4 °C. After intensive washing with incubation buffer, RNA-bound proteins were collected by addition of 35 μl of 1× Laemmli buffer and the pull-down material was subsequently analyzed by Western blot analysis by probing the membranes with a HuR-specific antibody. Equal input material was confirmed by Western blotting using the same antibody. After incubation with the secondary antibody, the immunopositive signals were visualized by ECL.

Cell cycle and apoptosis analysis

The analysis of cell cycle distribution and the sub-G_1 population of different colorectal carcinoma cells was performed with a FACSCanto II flow cytometer (Becton Dickinson, Heidelberg, Germany). Briefly, cells were seeded in 60 mm dishes and transfected with the relevant siRNAs as described before. At 24 h after siRNA transfection, the cells were trypsinized, centrifuged (300 × g for 5 min), resuspended in growth medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin before 4.0–6.0 × 10^5 cells were seeded on 60 mm petri dishes. 24 h later the cells were exposed to irradiation and a subsequent 24 h later trypsinized, washed in PBS and fixed overnight in absolute ethanol at −20 °C. After centrifugation (300 × g for 2 min), cell pellets were resuspended in 0.3 ml hypotonic buffer containing 50 μg/ml propidium iodide (Sigma–Aldrich); 0.1% sodium citrate; 0.1% Triton X-100 and 10 μg/ml RNase A and incubated for 30 min at 37 °C before measurement. Finally, cells were gated to exclude cell debris and analyzed by flow cytometry in linear mode by using the FACSDiva Software (Becton Dickinson).

Immunofluorescence staining and quantification of γH2AX/53BP1 foci formation

Analysis of residual DNA double-strand breaks (DSBs) was performed by counting of γH2AX/53BP1-positive nuclear foci. Colorectal carcinoma cells were subjected to siRNA transfection and 24 h later plated on microscope cover glasses in 12-well plates (neoLab Migge, Heidelberg, Germany). Cells were irradiated (0, 2, 6 Gy) after 24 h, fixed and permeabilized with 3.7% paraformaldehyde and 0.25% Triton X-100 (AppliChem, Darmstadt, Germany) in PBS for 10 min at 24 h after irradiation. After blocking in 5% bovine serum albumin (Applichem), staining was accomplished with anti-γH2AX (clone JBW301, #05-636, Millipore, Schwalbach, Germany) and anti-53BP1 (#100-304, Novus Biologicals, Cambridge, UK) primary and Alexa-labeled secondary antibodies (Alexa Fluor 594 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, Life Technologies, Darmstadt, Germany). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) solution (Life Technologies). Cover slips were mounted with Vectashield mounting medium (Alexis,