Biochemical Pharmacology 98 (2015) 51–68

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Resveratrol induces chemosensitization to 5-fluorouracil through up-regulation of intercellular junctions, Epithelial-to-mesenchymal transition and apoptosis in colorectal cancer

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ARTICLE INFO

Article history:
Received 7 July 2015
Accepted 20 August 2015
Available online 24 August 2015

Chemical compounds studied in this study:
5-Fluorouracil (5-Fluor-1*H*-pyrimidin-2,4-dion)
Resveratrol (*trans*-3,5,4'-Trihydroxystilben)

Keywords:
Colorectal cancer
Resveratrol
5-FU
Junctions
Claudin-2
E-cadherin

ABSTRACT

5-Fluorouracil (5-FU), a common chemotherapeutic agent used for the treatment of colorectal cancer (CRC), by itself has inadequate response rates; highlighting the need for novel and improved treatment regimens for these patients. Resveratrol, a naturally-occurring polyphenol, has been linked with chemosensitizing potential and anticancer properties; however, the underlying mechanisms for these effects remain poorly understood.

The effect of resveratrol in parental CRC cell lines (HCT116, SW480) and their corresponding isogenic 5-FU-chemoresistant derived clones (HCT116R, SW480R) was examined by MTT assays, intercellular junction formation and apoptosis by electron- and immunoelectron microscopy, nuclear factor-kappaB (NF-κB) and NF-κB regulated gene products by western blot analysis in a 3D-alginate microenvironment. Resveratrol blocked the proliferation of all four CRC cell lines and synergized the invasion inhibitory effects of 5-FU. Interestingly, resveratrol induced a transition from 5-FU-induced formation of microvilli to a planar cell surface, which was concomitant with up-regulation of desmosomes, gap- and tight junctions (claudin-2) and adhesion molecules (E-cadherin) expression in HCT116 and HCT116R cells. Further, resveratrol significantly attenuated drug resistance through inhibition of epithelial-mesenchymal transition (EMT) factors (decreased vimentin and slug, increased E-cadherin) and down-regulation of NF-κB activation and its translocation to the nucleus and abolished NF-κB-regulated gene end-products (MMP-9, caspase-3). Moreover, this suppression was mediated through inhibition of IκBα kinase and IκBα phosphorylation and degradation. Our results demonstrate that resveratrol can potentiate the anti-tumor effects of 5-FU on CRC cells by chemosensitizing them, inhibiting an EMT phenotype via up-regulation of intercellular junctions and by down-regulation of NF-κB pathway.

1. Introduction

Colorectal cancer (CRC) ranks among the third most frequent cancer in the world, affecting both men and women [1]. There is a tremendous clinical interest in improving therapeutic management of primary CRC, as well as advanced, metastatic cancers after surgery or chemotherapy, since ~50% of cases develop chemoresistance to standard of care chemotherapeutic drugs such as 5-fluorouracil (5-FU) [1,2]. Metastasis of tumor cells presents a malignant event that leads to spreading of cancer cells to other tissues, and often results in increased mortality [3]. In addition, conventional chemotherapies associate with

Abbreviations: AT, ambient temperature; BSA, bovine serum albumin; CRC, colorectal cancer; DMEM, Dulbecco's modified Eagle's medium; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; IKK, IκB kinase; 5-FU, 5-Fluorouracil; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; PARP, poly(ADP-Ribose) polymerase; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl-fluoride; Sirt1, Sirtuin-1; SDS–PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; TEM, transmission electron microscope; TGF, transforming growth factor.

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http://dx.doi.org/10.1016/j.bcp.2015.08.105
0006-2952/

severe side effects, including abdominal pain mucositis, as well as high expense [4]. These limitations highlight the need for development of novel anti-tumor agents that may enhance chemosensitivity of tumor cells and suppression of metastasis.

It has been reported that intercellular communication through junctional complexes, such as desmosomes, tight and gap junctions increases intercellular adhesion, thereby stabilizing tissue homeostasis [5,6]. In contrast, loss of such intercellular communication closely correlates with increased metastatic potential, acts as a promotor for tumor cell proliferation, detachment and invasion [5,7]. Epithelial cells in the intestine form a barrier separating the external environment from the inside of the body. Adherence junctions and desmosomes play a critical role in cell–cell adhesion in enterocytes and provide an adhesive force to ensure the integrity of the cellular layer, whereas tight junctions seal off these compartments [8]. These functional structures have a similar organization, whereby transmembrane adhesive components are bound to cytosolic adapter proteins providing a link to the cytoskeleton [9]. Tight junctions are composed of proteins from the claudin, occludin and IgG-like family of junctional adhesion molecules [10,11]. However, claudins are the determining structures for barrier properties [12]. Claudins are transmembrane proteins that contain two extracellular domains and one intracellular domain with N- and C-termini facing the cytoplasm [13]. Expression of claudin −1, −2, −3, −4, −7, −8, −12 and −15 have been identified in the colon, whereby in human colorectal cancer, claudin-1, −2, −3, −4, −7 expression levels have been found to be significantly upregulated [12,14–16]. Moreover, the expression of claudins is regulated by many factors, including epithelial–mesenchymal transition (EMT)-related transcription factors such as Snail, hormones and various cytokines [17–19]. In general, most claudins are epithelial tight junction proteins that are down-regulated by EMT factors such as Snail and Slug [3]. Recently, it has been recognized that cytoskeletal reorganization, acts as a mediator of physiological and pathophysiological tight junction regulation [8].

A hallmark for EMT is the functional loss of E-cadherin, which is associated with loss of adherence between the epithelial cells converting them to become more motile, invasive and acquire more mesenchymal characteristics [20,21]. Loss of polarity during EMT is associated with loss of tight junctions, a process which is thought to be mediated by Snail and Slug independently of E-cadherin regulation [22,23]. Indeed, enhanced tight junction formation has been observed only in weakly metastatic tumor cells, whereas highly metastatic cancer cells have markedly poor expression of tight junctions [5].

It is well recognized that the majority of solid and hematopoietic cancer cells have constitutively active NF-κB [24]. NF-κB plays an important role in cell proliferation and malignant transformation in different cells, through its binding to DNA target sites as homo- or heterodimers and by influencing downstream gene expression [25,26]. Pro-inflammatory cytokines, chemotherapeutic agents and radiation therapy, which induce apoptosis, also activate NF-κB and thus may mediate chemoresistance and radioresistance of cancer cells [27].

The natural polyphenol Resveratrol (*trans*-3,5,4′-Trihydroxystilben) is found in the skin of grapes, various berries and nuts, and possesses a wide spectrum of pharmacological properties, including anti-tumor metastasis activities [28,29]. Resveratrol is a potent natural activator of Sirtuin-1 (SIRT1), a nucleus related histone deacetylase class III [30]. Resveratrol further inhibits IκB kinase (beta)-mediated NF-κB activation [31–33]. In addition, several studies have shown that in cancer, resveratrol induces cell cycle arrest and apoptosis [34–36], inhibits EMT-associated cancer cell migration and invasion through the inhibition of the PI-3K/Akt/NF-κB signaling pathway, inhibition of TGF-β1 and inhibition of hedgehog signaling pathway [29,37,38].

In the present study, we investigated the effect of resveratrol individually, and in combination with 5-FU on various growth regulatory parameters and extensive characterization of underlying mechanisms in a series of CRC cell lines. Our data firstly reveal that the up-regulation of intercellular junctions and MET by resveratrol, via down-regulation of NF-κB activation is one of the principle mechanisms of inhibition of tumor growth and invasion, thereby sensitizing CRC cells to 5-FU and potentiating apoptosis. These findings highlight the potential possibility of using such natural, safe and relatively inexpensive compounds as potential adjunctive treatments in improving the overall treatment response of patients with CRC in future.

2. Material and methods

2.1. Antibodies

Polyclonal anti-claudin-2 was obtained from Abcam PLC (Cambridge, UK). Polyclonal antibodies to active caspase-3 were from R&D Systems, Inc., (Heidelberg, Germany). Monoclonal anti-pan-IκBα, anti-E-cadherin, anti-vimentin and anti-Slug were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal poly(ADP-ribose) polymerase (PARP) antibodies were purchased from Becton Dickinson (Heidelberg, Germany). Monoclonal anti-β-actin antibody was purchased from Sigma-Aldrich Chemie (Munich, Germany). Anti-phospho-specific p65 (NF-κB) /(Ser536), anti-phospho-specific p50 (NF-κB) and anti-phospho-specific IκBα (Ser-32/36) were obtained from Cell Technology (Beverly, MA, USA). Anti-IKK-α and anti-IKK-β antibodies were obtained from Imgenex (Hamburg, Germany). Alkaline phosphatase–linked sheep anti-mouse and sheep anti-rabbit secondary antibodies for immunoblotting were purchased from EMD Millipore (Schwalbach, Germany). Secondary antibodies used for fluorescence labelling were purchased from Dianova (Hamburg, Germany). Gold particle-conjugated secondary antibodies were purchased from Amersham (Braunschweig, Germany).

2.2. Growth media, chemicals and cytokines

Cell culture growth medium consisting of Dulbecco's modified Eagle's medium/Ham's F-12 (1:1), 10% fetal bovine serum (FBS), 1% amphotericin B solution, 1% penicillin streptomycin solution (10,000 IU/10,000 IU), 75 μg/ml ascorbic acid, 1% essential amino acids and 1% glutamine was obtained from Seromed (Munich, Germany). Epon was obtained from Plano (Marburg, Germany). Alginate and 5-Fluorouracil (5-FU) were purchased from Sigma (Munich, Germany). Resveratrol with purity greater than 98% was purchased from Sigma. A 100 mM stock solution of resveratrol (molecular weight 228.2) was prepared in ethanol and further diluted in cell culture medium to prepare working concentrations. The maximum final content of ethanol in cultures was less than 0.1%. This concentration was also used as a control. A 100 mM stock of 5-FU was prepared in absolute DMSO and stored at −20 °C. For treatment, the 5-FU stock solution was diluted in DMEM/F12 and added to cultures to achieve the desired concentration. The final concentration of DMSO was less than 1% of drug treatment. For treatment, 5-FU was diluted in DMEM and added to cultures to give the desired final concentration.

2.3. Cell lines and cell culture

Human HCT116 CRC cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). SW480 CRC cells were purchased from ATCC. We also generated 5-FU resistant derivatives