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Proliferation rate but not mismatch repair affects the long-term response of colon carcinoma cells to 5FU treatment

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ABSTRACT

The role of mismatch repair (MMR) in the response of colon carcinoma cells to 5-fluorouracil (5FU) is not well understood. In most of the *in vitro* studies only short-term response was investigated. We focussed here on the influence of MMR status on the mechanism of the short- and long-term response to clinically relevant 5FU concentrations by using isogenic or semiisogenic cell line pairs expressing/nonexpressing the hMLH1 protein, an important component of the MMR system.

We show that the lower survival of MMR-proficient than of MMR-deficient cells in the clonogenic survival assay is due to a more frequent early cell arrest and to subsequent senescence.

By contrast, the long-term cell growth after treatment, which is also affected by long-term arrest and senescence, is independent from the MMR status. The overall effect on the long-term cell growth is a cumulative result of cell proliferation rate-dependent growth inhibition, apoptosis and necrotic cell death. The main long-term cytotoxic effect of 5FU is the inhibition of growth while apoptosis and the necrotic cell death are minor contributions.

1. Introduction

5-Fluorouracil (5FU) is widely used for chemotherapy of cancers of different organs including colorectum, breast and lung [1]. The response rates of colorectal carcinomas are 20–30%, in combination with irinotecan [2] or oxaliplatin [3] they reach 40–50%. It is therefore crucial to establish the determinants of susceptibility to 5FU which would allow to select the population of patients who would benefit from the treatment.

Apoptosis, necrosis and proliferation inhibition have been identified as the main cellular responses to treatment with 5FU. Apoptosis is induced in colon carcinoma cells within 1–3 days in a p53-dependent manner [4,5]. This is associated with a lesser clonogenic survival of cell lines with functional p53 as compared to the isogenic p53-defective counterparts [5].

Furthermore, focal necrotic foci appear in mice tumours within 24 h after start of 5FU treatment and are evident for several days after completion of treatment [6]. Later, no massive increase of necrosis is observed.

Several molecular mechanisms have been associated with the cytotoxic effects. 5FU is known to irreversibly inhibit thymidylate synthase which synthesizes thymidine monophosphate, the sole *de novo* source of thymidylate. The lack of thymidine monophosphate results in inhibition of DNA synthesis and consequently in inhibition of cell proliferation (reviewed in [1]). High activity of thymidylate synthase correlated with poor short-term response to 5FU in several colorectal cancer cell lines [7].

On the other hand, in some human carcinoma cell lines higher BrdU incorporation correlated better with a short-term sensitivity to 5FU than thymidylate synthase activity [8,9], and cell lines with a small S-phase population were found to be less responsive to 5FU than those with a larger S-phase population [10]. Indeed, the value of thymidylate synthase expression as a clinical predictor of response has been recently questioned [11].

The importance of S-phase duration and of cell proliferation rate as response predictors is underscored by the observation that low precursor incorporation and slow-down of the S phase, which is controlled by the Chk1 kinase, were associated with tumour cell protection [12]. When Chk1 was downregulated (or inhibited by UCN-01), and the cells exited the S-phase arrest after 5FU treatment, they entered mitotic catastrophe [13,14], which enhanced the cytotoxicity.

Incorporated 5FU is removed from the DNA by the short patch base excision repair (BER), the long patch BER [15] and by the mismatch repair system (MMR) [16]. The cytotoxicity is postulated to be enhanced by the removal of 5FU and by the resulting DNA fragmentation. How important are the relative contributions of BER and of MMR to 5FU cytotoxicity has not been investigated (reviewed in [17]).

The model in which 5FU cytotoxicity is due to the excision of fluorouracil from DNA is opposed by the observation that the

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cytotoxicity correlates with the levels of 5FU retained in the DNA [18]. These latter data suggest that the persistence of 5FU in the DNA rather than the excision of uracil is responsible for 5FU-mediated growth inhibition. According to this concept, the retention of 5FU in the DNA is cytotoxic and base excision repair of 5FU initiated by BER protects cells from 5FU effects [18].

The question of how MMR status affects the response to 5FU is of particular clinical interest. The base of the hypothesis that MMR affects the response to 5FU are the consistent results of clonogenic and MTT assays which show that MMR-proficient colon carcinoma cells are more susceptible to 5FU than MMR-deficient ones [19,20]. Since MMR deficiency is easily detectable in human tumours by RT-PCR as microsatellite instability or by immunohistochemistry as lack of hMLH1 protein expression, the MMR-proficient and MMR-deficient tumours can be rapidly differentiated and corresponding patients' groups identified.

Clinical reports on the effect of MMR status on the response of colorectal cancer to adjuvant 5FU therapy are, however, conflicting: certain authors reported that patients with MMR-deficient tumours do not benefit from 5FU treatment [21–25] while others found either no effect of MMR status on the response at all [26,27] or that MMR defect was a predictor of benefit from 5FU therapy, when compared with the MMR-deficient nontreated group [28–30]. A more refined recent study indicates that 5FU treatment improves survival of patients with suspected HNPCC but not with sporadic MMR-deficient cancers [31].

The objective of the present work was to clarify which cellular reactions to 5FU determine its long-term cytotoxicity and how the MMR-status influences it. Due to the multiple effects of 5FU, like proliferation inhibition, DNA repair activation, apoptosis and necrosis, the details of the mechanism of cytotoxicity can be clarified only in a very well-defined cell system. We carried out the analysis in a recently established isogenic cell system [32], in which the only difference between the clones is the presence/absence of the hMLH1 molecule, as well as in the previously described semiisogenic cell system HCT116/HCT116 + chr3/HCT116 + chr2 [33].

In this reductionist setup, in which the MMR status and the proliferation rate were separately taken into consideration as independent variables, we show that in contrast to the short-term response to 5FU, which is affected by the MMR status, the long-term response is MMR-status independent and is affected mainly by the proliferation rate.

2. Materials and methods

2.1. Cell lines and stable hMLH1 transfectants

HCT116 (MMR-deficient), HCT116 + chr2 (MMR-deficient), HCT116 + chr3 (MMR-proficient) cells were a kind gift from Dr. C.R. Boland (Baylor University Medical Center, Dallas, USA). The culture medium for HCT116 + chr3 and HCT116 + chr2 cells contained 0.4 mg/ml G418. HCT116Bax$^{-/-}$ cell line was a kind gift of Dr. Bert Vogelstein.

hMLH1-expressing stable clone 43 or the mock transfectants were derived from the HCT116 cell line transfected with pcDNA3.1-myc-hMLH1 plasmid or with an empty plasmid and were extensively characterised recently [32].

2.2. Transient transduction with Adv-hMLH1

HCT116 cells were incubated with adenovirus-hMLH1 (Adv-hMLH1) or with adenovirus-LacZ (Adv-LacZ) for 90 min at 37 °C in medium without serum. Then 10% FCS was added. After 48 h cells were replated for clonogenic assay.

2.3. Clonogenic assay

Ten cells/cm^2 were seeded, 24 h later they were treated with 10–30 μM 5FU, washed after 2 days with PBS and grown in drug free medium for 14 days. Cells were fixed with formamide/glutaraldehyde (2%/0.2%), and stained with crystal violet. Colonies with more than 50 cells were counted.

2.4. 5FU treatment of cells

Unless mentioned otherwise, the cells were treated with 30 μM 5FU (GRY Pharma, Radebeul, Germany) for 2 days, then washed with PBS and then grown in drug-free medium.

2.5. Determination of cell growth

4×10^4 cells/cm^2 were seeded, 24 h later they were treated with 5FU for 2 days, PBS-washed and grown in drug free medium for 16 days. For growth in the absence of 5FU, 10^4 cells/cm^2 were seeded. At indicated time points, cells were trypsinized, floating and adherent cells were pooled, stained with trypan blue and counted.

2.6. Western blotting

Two days after 5FU treatment, adherent and floating cells were harvested, washed with cold PBS and lysed in cold lysis buffer [34] for 15 min on ice. Lysates were cleared by centrifugation. 20 μg of protein per lane were separated on SDS polyacrylamide gel and electroblotted onto Immobilon-P membrane. The membranes were blocked with 5% nonfat dry milk in 0.1% Tween 20 in TBS for 1 h, then incubated overnight at 4 °C with the corresponding primary antibody. The following primary antibodies were used: mouse anti-PARP, mouse anti-hMLH1 (both from BD Transduction Laboratories, Lexington, KY, USA; mouse anti-tubulin (Sigma–Aldrich Chemie, Taufkirchen, Germany), rabbit anti-Bax (Santa Cruz). Goat anti-mouse (IgG + IgM)- or goat anti-rabbit IgG peroxidase-HRP conjugates (Dianova, Hamburg, Germany) were used as second antibodies. After detection using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) the images were evaluated in Image Reader LAS 4000 instrument (Fujifilm Corporation, Tokyo, Japan).

2.7. Detection of apoptosis and necrotic cell death

The extent of apoptosis was estimated as the ratio of the intensity of the cleaved 85 kDa PARP band/intensity of 85 kDa PARP band + intensity of 115 kDa PARP band. Necrotic cell death was determined by measuring the release of lactate dehydrogenase (Homogenous Membrane Integrity Assay kit, CytoTox-One, Promega Corporation, Madison, WI, USA) and expressed as relative fluorescence units (RFU)/10^2 cells. For determination of the time course, apoptosis or necrotic cell death were determined at day 2 (1–2 days time window), then the medium was changed and the determination was repeated at day 6 (3–6 days time window), then the medium was changed and the measurement was carried out at day 10 (7–10 days time window).

2.8. Inhibition of caspases

Cells were preincubated with the broad-spectrum caspase inhibitor Q-VD-OPh (Calbiochem, Darmstadt, Germany) for 1 h at the concentration of 5 μM and then 5FU was added. After removal of 5FU and at every change of medium fresh inhibitor was added.

2.9. Immunohistochemistry

For detection of nuclear condensation, cytospins were fixed in ice-cold methanol and stained with DAPI. For determination of senescence, acid β-galactosidase was detected. For monitoring of BrdU incorporation, cells were incubated for 60 min with 10 μM BrdU. Mouse anti-BrdU antibody (BD Pharmingen No. 33284X) followed by a biotin-labelled donkey anti-mouse IgG (Dianova, Germany) and Cy3-labelled streptavidine (Dianova) were used for detection. Nuclei were counterstained with DAPI and coverslips mounted with Fluoromount.

2.10. Flow cytometry

Floating and trypsinized cells were fixed in ice-cold 70% ethanol at −20 °C for 2 h and stained in PBS with propidium iodide, 0,1% Triton X 100 and RNaseA. Cell cycle was analyzed on a FACSCalibur (Becton Dickinson) using ModFit LT2 software (Verity Software House, Topsham, ME, USA) for evaluation.

For BrdU detection, cells were incubated for 30 min with 10 μM BrdU. FITC-conjugated anti-BrdU antibody (BD Pharmingen, No. 33284X) was used for detection.

3. Results

3.1. The better clonogenic survival of MMR-deficient than MMR-proficient cells is associated with less frequent single cell arrest and is independent from apoptosis

Clonogenic assay reflects the capacity to form colonies shortly after removal of 5FU, i.e. it represents the "frozen" short-term