Cancer Letters 284 (2009) 95–101

Contents lists available at ScienceDirect

Cancer Letters

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

Methotrexate-induced senescence in human adenocarcinoma cells is accompanied by induction of p21$^{waf1/cip1}$ expression and lack of polyploidy

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ARTICLE INFO

Article history:
Received 20 January 2009
Received in revised form 8 April 2009
Accepted 9 April 2009

Keywords:
High dose methotrexate
Colorectal adenocarcinoma
Growth arrest
Accelerated senescence
Multinucleation

ABSTRACT

Human colorectal adenocarcinoma C85 cells, treated with high dose methotrexate (1 μM; IC_{50} = 51 nM), undergo accelerated senescence, as the cells (i) are growth arrested at the G_1 and S phases of the cell cycle, (ii) are SA-β-galactosidase-positive, (iii) show induced expression of p21$^{waf1/cip1}$ and decreased expression of p16^{INK4a}, and (iv) show DNA synthesis continued at the reduced level. The fraction of C85 cells with DNA content higher than 4 N is maintained at the same level (14%) in cells untreated, as well as regrown after the treatment. Multinucleation is found as the main karyotypic abnormality.

1. Introduction

Methotrexate (MTX) is a classic antifolate used in cancer chemotherapy for nearly 60 years. Antiproliferative and cytotoxic properties of methotrexate are attributed to its depletive effect on the intracellular pool of reduced folates and a subsequent abrogation of thymidylate and purine synthesis [1]. Imbalance in DNA precursor pools results in turn in accumulation of DNA damage, assumed to constitute the primary manifestation of methotrexate cytotoxicity [2,3]. In various cellular systems exposure to the drug was shown to induce apoptotic cell death [4], and in a number of cases it was found to induce differentiation [5,6]. Treatment with high dose methotrexate was also found to induce senescence-like growth arrest, so far documented in detail only for human breast cancer MCF-7 cells [7]. Despite an unequivocal success of methotrexate, resistance to this drug constitutes a major limitation of its anticancer application [8].

Cellular senescence reflects the stage of permanent growth arrest, occurring as a response to diverse stressful conditions [9]. Depending on the nature of stress signal, three main forms of senescence are distinguished: (i) replicative senescence induced by eroded telomeres, (ii) premature senescence induced by DNA damaging agents and (iii) oncogene-induced senescence resulting from hypermitogenic stimulation [10,11]. Despite a variety of triggering factors, in all forms of senescence activation of DNA damage response pathways leads to ultimate induction of a common senescent phenotype [12]. Senescence is postulated to constitute an effector program serving to eliminate cells with aberrant genetic material and thus to pose a natural barrier against tumorigenesis [13]. It was assumed until recently that cancer, in contrast to normal, cells cannot undergo senescence. However, radiation and drug treatment were proved to be capable of inducing cessation of proliferation, leading to a senescence-like phenotype of cancer cells (also termed stress-induced senescence, premature or accelerated senescence), which is considered an important factor contributing to the outcome of cancer therapy [14,15].

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doi:10.1016/j.canlet.2009.04.015

Treatment of human colorectal adenocarcinoma C85 cells with 1 μM methotrexate was previously reported to result in the cell cycle G_1-phase arrest, cell flattening, increase in granularity and changes in spectral properties [16], considered characteristic for cellular senescence. The notion that the applied high dose methotrexate exposure induces in C85 cells senescence is further pursued in the present study, showing that although C85 cells are capable of undergoing apoptosis, accelerated senescence, dependent on p53/$p21^{waf1/cip1}$ signaling pathway, constitutes the prevalent response.

2. Materials and methods

2.1. Cell culture

Human colorectal adenocarcinoma C85 cells, originating from primary, untreated tumor [17], were maintained in RPMI 1640 medium (Cambrex), supplemented with 10% Fetal Bovine Serum (Sigma–Aldrich), 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Invitrogen Life Sciences), and growth under such conditions was considered regular or control. Cells were exposed to various doses of methotrexate (Schircks Laboratories), provided in detailed descriptions of particular experiments. Regrowth, i.e. recovery of C85 cells after 48 h exposure to 1 μM methotrexate, took place in the regular medium for 96 h, with the exception of bromodeoxyuridine incorporation assay, where the time point of 48 h recovery was added.

2.2. Cytotoxicity assay

For MultiTox-Fluor Multiplex Cytotoxicity assay (Promega) C85 cells were exposed for 48 h to methotrexate concentrations ranging between 10^{-9} and 10^{-5} M, in 10× increments. The assay is based on relation of intra-cellular (live cell response) and extra-cellular (dead cell response) protease activities, measured simultaneously with membrane permeant and membrane impermeant substrates. The assay was performed in triplicates, in 96-well plates with 5×10^4 cells adhered per well, as recommended by the manufacturer. Fluorescence was measured on Perkin–Elmer LS-5B Luminescence Spectrophotometer. The results are calculated as percentage of the assay response under control conditions.

2.3. Senescence-associated β-galactosidase (SA-β-gal) activity assay

SA-β-gal activity was detected colorimetrically as earlier described [18]. The images were taken with Olympus IX70 microscope.

2.4. Western blotting

Lysates preparation, blotting and chemiluminescent signal detection were performed as previously described [16], using the following combinations of antibodies: (i) anti-p16 (Santa Cruz Biotchnology) and peroxidase-conjugated anti-mouse immunoglobulins (Sigma–Aldrich), (ii) anti-p21 (Santa Cruz Biotechnology) and peroxidase-conjugated anti-rabbit immunoglobulins (Sigma–Aldrich), and (iii) anti-actin and peroxidase-conjugated anti-mouse IgM (both from Calbiochem).

2.5. Bromodeoxyuridine (BrdU) and 4′,6-diamidine-2-phenyl indole (DAPI) labeling assays

BrdU incorporation and DAPI labeling were determined with cells attached to coverslips and either exposed to 1 μM methotrexate for various time points or exposed and subsequently regrown. BrdU (Sigma–Aldrich) was added to the culture medium 16 h before analysis (10 μM final concentration). BrdU was detected in ethanol-fixed cells using anti-BrdU primary (Becton Dickinson), and Alexa Fluor 488-conjugated anti-mouse secondary (Molecular Probes), antibodies. For nuclei visualization cells were stained with 30 nM DAPI solution (Sigma–Aldrich). The images were taken at 200× magnification with Nikon Eclipse 50i fluorescence microscope, applying Dako Fluorescent Mounting Medium (Dako) to mount the coverslips. To estimate the number of BrdU-positive cells, at least 800 cells from randomly selected fields of each of three independent experiments were counted for each indicated time point. The results are presented as percentage of BrdU-positive cells in relation to the total cell number determined based on DAPI staining. DAPI-stained cells were also used to investigate nuclear morphology. The average percentage (±SD) of multinuclear cells was calculated from three separate experiments with at least 600 cells scored from randomly selected fields.

2.6. DNA content analysis

DNA content was measured in cells either exposed for 48 h to 1 μM methotrexate or exposed and subsequently regrown, employing the method of Nicoletti et al. [19]. The analysis was performed on cells detached by trypsinization, washed three times with phosphate buffered saline and resuspended in hypotonic propidium iodide solution, at 3×10^5 cells/ml density. Fluorescence was

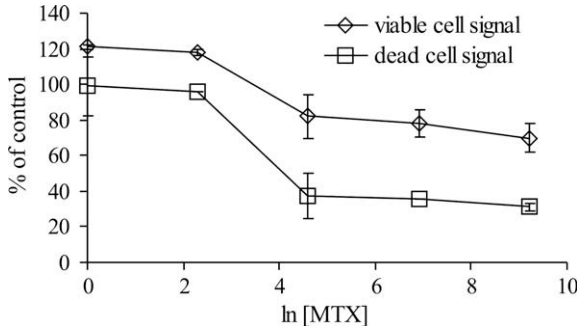

Fig. 1. Methotrexate (MTX) toxicity towards C85 cells estimated by MultiTox-Fluor Multiplex Cytotoxicity assay (Promega). Each point represents mean value of live- or dead-cell-specific protease activity, denoted viable cell signal and dead cell signal, respectively. ±SD for $N = 3$ is marked.