BBA - General Subjects 1862 (2018) 557–566

Contents lists available at ScienceDirect

BBA - General Subjects

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

Modulation of Mcl-1 transcription by serum deprivation sensitizes cancer cells to cisplatin

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ARTICLE INFO

Keywords:
Apoptosis
Cancer
Chemotherapy
Mcl-1
Serum deprivation

ABSTRACT

Background: The development of approaches that increase therapeutic effects of anti-cancer drugs is one of the most important tasks of oncology. Caloric restriction in vivo or serum deprivation (SD) *in vitro* has been shown to be an effective tool for sensitizing cancer cells to chemotherapeutic drugs. However, the detailed mechanisms underlying the enhancement of apoptosis in cancer cells by SD remain to be elucidated.

Methods: Flow cytometry, caspase activity assay and western blotting were used for cell death rate evaluation. Western blotting, gel-filtration, siRNA approach and qRT-PCR were used to elucidate the mechanism underlying cell death potentiation upon SD.

Results: We demonstrated that SD sensitizes cancer cells to treatment with chemotherapeutic agent cisplatin. This effect is independent on activation of caspases-2 and -8, apical caspases triggering apoptosis in response to genotoxic stress. SD potentiates cell death via downregulation of the anti-apoptotic protein Mcl-1. In fact, SD reduces the Mcl-1 mRNA level, which consequently decreases the Mcl-1 protein level and renders cells more susceptible to apoptosis induction via the formation of apoptosome.

Conclusions: Mcl-1 protein is an important regulator of sensitivity of cancer cells to apoptotic stimuli upon SD.

General significance: This study identifies Mcl-1 as a new target for the sensitization of human cancer cells to cell death by SD, which is of great significance for the development of efficient anti-cancer therapies.

1. Introduction

DNA damaging agent-based therapy is one of the most commonly used strategies in anti-cancer treatment. DNA damage triggers apoptosis, a cell death process, which is essential for the normal functioning of the organism and represents an important oncosuppressive function. However, there are strong indications that single-agent chemotherapy is not sufficiently effective against cancer, because tumor cells are genetically heterogeneous and highly adaptable. Therefore, the combinations of different treatment approaches are intensively investigated in order to improve the anti-cancer therapies. A number of pro- and anti-apoptotic proteins, including Bcl-2 family members, tightly regulate initiation of apoptosis [1]. Interference with their expression levels and functions can shift the threshold of apoptosis induction. One of the potential targets, downregulation of which leads to the enhancement of apoptosis in cancer cells, is Mcl-1, an anti-apoptotic member of the Bcl-2 protein family [2]. This family comprises > 20 proteins, including both proapoptotic (e.g. Bax and Bak) and anti-apoptotic members (e.g. Bcl-2, Bcl-xL, and Mcl-1) [3]. The balance between these pro- and anti-apoptotic proteins defines the susceptibility of cells to apoptosis. Mcl-1 is crucially important for binding several proapoptotic proteins, such as Bim and Bak, which leads to apoptosis inhibition. Consistent with its anti-apoptotic functions, upregulation of Mcl-1 expression has been detected in both hematological and solid tumors [4,5]. It has also been shown that Mcl-1 is localized at the mitochondrial matrix, maintaining normal functioning of mitochondria (i.e. regulates its fusion and promotes the assembly of ATP synthase oligomers) [6]. In contrast to other main anti-apoptotic proteins of the Bcl-2 family, namely Bcl-xL and Bcl-2, Mcl-1 protein is characterized by a very short half-life. Mcl-1 contains two PEST (proline [P], glutamic acid [E], serine [S], and threonine [T]) motives, which promote Mcl-1 proteasomal degradation [7]. Mcl-1 expression is regulated by many extracellular stimuli, and stimulation of several oncogenic signaling pathways leads to Mcl-1 upregulation [8]. Thus, Mcl-1 could be

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https://doi.org/10.1016/j.bbagen.2017.11.021
Received 21 September 2017; Received in revised form 19 November 2017; Accepted 29 November 2017
Available online 02 December 2017
0304-4165/

regarded as a first line of anti-apoptotic defense in cancer cells.

One of the potentially promising approaches in anti-cancer therapy is a combination of treatment with anti-cancer drugs with so-called short-term calorie restriction [9,10]. Among different approaches in vitro, serum deprivation (SD) has been shown to serve as a model of short-term calorie restriction and simultaneously to be efficient in sensitizing cancer cells to anti-cancer drugs due to the similarity of molecular pathways implicated in cellular response to this type of stress [11,12]. SD is typically administrated via decrease of the content of fetal bovine serum (FBS), which is commonly used to supplement growth media. However, the reports on the molecular mechanisms of SD action on sensitization of cancer cells to apoptosis are still contradictory. Various mechanisms of SD-mediated apoptosis have been suggested. In particular, primary neurons subjected to SD have been reported to undergo massive apoptosis through a caspase-2-dependent mechanism [13]. It has also been shown that in colorectal and breast cancer cells the proapoptotic proteins Bax and Puma mediate SD-induced apoptosis. This effect was counteracted by p21 and reinstated by Bcl-xL inhibition [14]. In experiments on tumor and normal cells, Shi et al. demonstrated selective sensitization of cancer cells by SD to cisplatin. SD led to the activation of AMPK-signaling in both tumor and normal cells. In contrast, the ATM/Chk2/p53 signaling pathway was activated only in tumor cells upon SD action. Treatment with cisplatin additionally stimulated the ATM/Chk2/p53 signaling pathway and thereby induced apoptosis in tumor cells [15].

Our aim was to uncover molecular mechanisms of sensitization effects mediated via SD on apoptosis using several cancer cell lines of different origin. We showed that SD sensitizes cancer cells to cisplatin-induced apoptosis independently of initiator caspases-2 and -8. Importantly, we found that Mcl-1 is a key regulator of SD-induced sensitization of cancer cells to apoptosis. Furthermore, it was demonstrated that this regulation occurs at the transcriptional level of regulation of Mcl-1 expression. Taken together, our findings shed light on the mechanisms underlying promotion of apoptosis in human cancer cells by SD, paving the way for development of more efficient approaches to the sensitization of cancer cells to chemotherapeutic agents.

2. Material and methods

2.1. Reagents

Cisplatin was purchased from Teva; Inhibitors MG-132, U0126, Q-VD-Oph were purchased from Sigma; A-1210477 was from Active Biochem and BSA was from Amresco. MG-132 was used at concentration of 200 nM; U0126 and A-1210477 were used at concentration of 10 μM, Q-VD-Oph was used at concentration of 20 μM.

2.2. Antibodies

The following primary antibodies were used: anti-cleaved PARP (#9541), anti-vinculin (#13901); anti-caspase-3 (#9662), anti-cleaved caspase-3 (#9661), anti-Bcl-2 (#4223), anti-Bcl-xL (#2764), anti-Mcl-1 (#5453), anti-p38 MAPK (#8690), anti-p44/42 MAPK (Erk1/2) (#4695), anti-Phospho-p38 MAPK (#4511), anti-Phospho-p44/42 MAPK (Phospho-Erk1/2) (#4370) – all from Cell Signaling; anti-GAPDH (#2275) – from Trevigen; anti-caspase-2 (#611022) – from BD Biosciences; anti-caspase-8 (ADI-AAM-118-E) – from ENZO Life Sciences; anti-caspase-9 (M054–3) – from MBL. The following secondary antibodies were used: Anti-rabbit IgG, HRP-linked (#7074) and Anti-mouse IgG, HRP-linked (#7076) – both from Cell Signaling.

2.3. Cell culture and experimental procedures

The epithelioid cervix carcinoma cell line HeLa and the human epithelial ovarian carcinoma cell lines Caov-3, Skov-3, Caov-4 wild-type and Caov-4 expressed caspase-2 shRNA were used in this study and the latter one was described earlier [16]. Cells were grown in a CO_2 incubator (5% CO_2) in DMEM containing 4.5 g/L glucose (Gibco), with 10% FBS (Gibco) at 37 °C, in the presence of a mixture of antibiotics and antimycotics (Gibco). Cells in the logarithmic growth phase were used for experiments. Before the treatment with cisplatin the culture medium was removed and cells were washed with PBS. Fresh serum-containing medium (DMEM supplemented with 10% FBS, 1% mixture of antibiotics and antimycotics) or FBS-free medium were added to cells. Then cells were immediately treated with 70 μM of cisplatin and incubated for indicated time points. If chemical inhibitors were used, they were added to the fresh medium an hour before the treatment with cisplatin. Cultivation in FBS free medium is designated as SD condition. If any additional compound was added, corresponding control cells received the same portion of respective solvent.

2.4. FACS analysis

After the indicated time of treatment, cells were collected by 0.05% trypsin-EDTA (Gibco) and transferred to a conditioned medium. The cells were centrifuged (300 rcf, 5 min, 4 °C) and washed twice with ice-cold PBS (Paneco). Then, cells (0.2×10^6) were resuspended in 200 μL of annexin-binding buffer (BD Biosciences) and 2 μL of annexin V-FITC (Invitrogen) were added. Then samples were incubated in the dark at room temperature for 15 min. Next, 5 μL of propidium iodide (50 μg/mL) (BD Biosciences) were added to each sample. After 5 min of incubation in the dark at room temperature the cells were analyzed by flow cytometry with BD FACS Canto II Analyzer™ (BD Biosciences).

2.5. Gel electrophoresis and western blot analysis (WB)

After the indicated time of treatment, cells were detached with cell-scraper in the conditioned medium. The cells were centrifuged (300 rcf, 5 min, 4 °C) and washed twice with ice-cold PBS (Paneco). Then, cell pellet was lysed in RIPA buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, cOmplete™ Protease Inhibitor Cocktail (Roche)) for 20 min on ice. Lysates were centrifuged (16,000 rcf, 20 min, 4 °C) and supernatants were used for further analysis. After protein concentration assay (with BCA Protein Assay Kit, Thermo Scientific) equal amounts of proteins (20–35 μg of total protein) from each probe were mixed with Laemmli loading buffer. These samples were boiled for 5 min at 95 °C, resolved by electrophoresis on SDS-PAGE and transferred to nitrocellulose membranes using Mini Trans-Blot® Cell (Bio-Rad). Then the membranes were blocked with 5% nonfat milk-TBS solution for 1 h at room temperature and washed three times in TBS for 5 min. The membranes were incubated with primary antibodies overnight at 4 °C. After three washes in TBS-containing 0.05% Tween-20, the membranes were incubated with secondary antibodies for 1 h at room temperature, washed four times in the TBS and visualized with either ECL (Promega) or SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific) on Molecular Imager®ChemiDoc™ (Bio-Rad). Densitometric analysis was performed using the ChemiDoc MP.

2.6. Gel filtration

Cells ($\sim 50 \times 10^6$) treated with 70 μM cisplatin with or without SD, were detached from the plates with a cell-scraper, washed three times in ice-cold PBS and then lysed for 30 min on ice in 2 mL of lysis buffer (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, cOmplete™ Protease Inhibitor Cocktail (Roche)). Lysates of cells were centrifuged (16,000 rcf, 25 min, 4 °C). 20 μL of the supernatant were collected to determine the protein concentration, 20 μL of supernatant was analyzed by WB and the rest was subjected to gel filtration. Gel filtration was performed with fast protein liquid chromatography on the ÄKTA FPLC explorer system (GE Healthcare) and 10/300 GL column with Superose 6 (GE Healthcare,