Cancer Letters 385 (2017) 75–86

Contents lists available at ScienceDirect

Cancer Letters

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

Original Article

Doxorubicin anti-tumor mechanisms include Hsp60 post-translational modifications leading to the Hsp60/p53 complex dissociation and instauration of replicative senescence

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ARTICLE INFO

Article history:
Received 29 July 2016
Received in revised form
26 October 2016
Accepted 28 October 2016

Keywords:
Doxorubicin
Hsp60
Acetylation
Ubiquitination
p53
Replicative senescence

ABSTRACT

The chaperone Hsp60 is pro-carcinogenic in certain tumor types by interfering with apoptosis and with tumor cell death. In these tumors, it is not yet known whether doxorubicin anti-tumor effects include a blockage of the pro-carcinogenic action of Hsp60. We found a doxorubicin dose-dependent viability reduction in a human lung mucoepidermoid cell line that was paralleled by the appearance of cell senescence markers. Concomitantly, intracellular Hsp60 levels decreased while its acetylation levels increased. The data suggest that Hsp60 acetylation interferes with the formation of the Hsp60/p53 complex and/or promote its dissociation, both causing an increase in the levels of free p53, which can then activate the p53-dependent pathway toward cell senescence. On the other hand, acetylated Hsp60 is ubiquitinated and degraded and, thus, the anti-apoptotic effect of the chaperonin is abolished with subsequent tumor cell death. Our findings could help in the elucidation of the molecular mechanisms by which doxorubicin counteracts carcinogenesis and, consequently, it would open new roads for the development of cancer treatment protocols targeting Hsp60.

Introduction

Replicative senescence (RS) or cellular senescence has been described as a state reached by normal mammalian fibroblasts cultured *in vitro* after a limited number of divisions [1]. In this state, senescent cells cannot divide and become un-responsive to growth signaling and resistant to apoptosis. Senescent cells show a flattened and enlarged shape and an increase in senescence-associated β-galactosidase (SA-β-gal) activity [2]. Cytoskeletal proteins may be involved in RS, for instance vimentin, since this protein is highly expressed in senescent fibroblasts [3].

Since cancer cells proliferate indefinitely, a requisite for their immortalization must be bypassing the physiological program that leads to RS. Abundant data support the notion that RS is a natural

Abbreviations: DMSO, dimethylsulfoxide; ELISA, enzyme linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GUSB, betaglucuronidase; HDAC, histone deacetylases; H2AX, H2A histone family member X; HPRT1, hypoxanthine phosphoribosyltransferase 1; Hsps, heat shock proteins; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazolium bromide; OD, optical density; RS, replicative senescence; SA-β-gal, senescence-associated β-galactosidase; Sirt3, sirtuin 3; 17AAG, 17-(Allylamino)-17-demethoxygeldanamycin.

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http://dx.doi.org/10.1016/j.canlet.2016.10.045
0304-3835/

barrier against tumorigenesis [4,5]. Stress-induced premature senescence is a program executed by cells in response to chemotherapy, and RS induced by DNA-damaging anticancer drugs is one of the key determinants of successful chemotherapy [6].

Heat shock proteins (Hsps) are highly expressed in a variety of cancer cells and are essential to their survival contributing to tumor cell propagation, metastasis, and protection against apoptosis [7,8]. Several Hsps function as molecular chaperones for other proteins to prevent their aggregation after environmental stress, for example. Molecular chaperones participate in the response to anti-cancer drugs [9,10] and are intensively studied as therapeutic targets and as diagnostic and/or prognostic markers in many types of cancer, for example breast cancer, osteosarcomas [11], ovarian carcinoma [12], and pancreatic carcinoma [13]. As stated above, the senescence program seems to represent one of the major breaks on cancer emergence and it has been demonstrated that high levels of chaperones play an important role in suppressing the senescent program, keeping the p53 signaling under control and thus allowing cancer cells to proliferate [14]. In fact, specific down-regulation of Hsp70 leads to rapid senescence of various cancer cell lines [15], and human neuroblastoma cells displayed senescence-like characteristics after inhibition of Hsp90 with tanespimycin (17AAG) [16]. The mitochondrial chaperonin Hsp60 also named HSPD1 [17] was found in multiple subcellular sites and function in the folding and intracellular trafficking of many proteins [18,19]. Hsp60 has been found elevated in a large number of human carcinomas, which opens novel perspectives for cancer diagnosis and therapy targeting Hsp60 [20,21]. The chaperonin can activate the immune system [22] and can have both, pro-survival and pro-death functions, depending on tissue, cell type, and apoptosis inducers [23].

Senescence and apoptosis are alternative cell fates: in some cases apoptosis is a response to intense stress while senescence is a consequence of mild damage [4]. For example, doxorubicin, an anthracycline antitumor drug widely used in clinical chemotherapy, induces senescence at low doses and apoptosis at high doses in breast cancer cells and in neonatal rat cardiomyocytes [24,25,26]. Hsp60 over-expression suppressed doxorubicin-induced apoptosis in cardiomyocytes [27], and doxorubicin-induced apoptosis in HeLa cells triggering Hsp60 up-regulation [23]. To the best of our knowledge no data are available that would show a direct participation of Hsp60 in RS. Here we investigated if sub-apoptotic doses of doxorubicin have an impact on the tumor-favoring Hsp60 action and, thereby, produces anti-tumor effects via the induction of RS in a human lung mucoepidermoid cell line.

Materials and methods

Cell culture and treatment conditions

The human lung mucoepidermoid cell line NCI-H292 was obtained from the American Type Culture Collection. Cells were routinely propagated and maintained in Roswell Park Memorial Institute medium (RPMI-1640, Sigma Aldrich, Milan, Italy) with 10% heat-inactivated Fetal Calf Serum (FCS, Life Technology, Milan, Italy), supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell line was grown as monolayers attached to 25 cm^2 culture flasks and cultured at 37 °C, 5% CO_2 in a humidified incubator. Doxorubicin hydrochloride (Sigma Aldrich) was prepared as an 8 μM stock solution in sterile water, stored at −20 °C and freshly dissolved immediately before use. Working dilutions were made in sterile water and were added to the complete cell culture medium at the appropriate concentrations. Twenty-four hours after seeding, when the cells reached 70% confluency, the cultures were treated with a range of concentrations of doxorubicin (range 5–1280 nM) for 5 days and untreated cells (UT) were maintained as control. After that, the cell culture medium was replaced with fresh drug-free complete medium and cells were left to grow further for 24 h, before harvesting them for testing. Cells were routinely photographed before and after treatment to record morphological changes occurring in the cells, using an inverted light microscope equipped with phase contrast rings (LEICA DM-IRB, Leica Microsystem, Milan, Italy). Measurements of the area of the cell were done and evaluated using ImageJ Free software (NIH, Bethesda, MD).

MTT assay

The viability of NCI-H292 cells treated with doxorubicin was measured using 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazolium bromide (MTT) obtained from Sigma Aldrich. The assay was performed as described [28]. Briefly, 5×10^3 NCI-H292 cells were plated in 200 μl of complete medium per well in 96-well plates and treated with a series of doses of doxorubicin for 24, 48, or 72 h, or 5 days. MTT was dissolved in fresh medium and added to the cell cultures at a final concentration of 0.5 mg/ml. Following a 2 h incubation period, the converted dye was solubilized in 200 μl of dimethylsulfoxide (DMSO)/well and optical density (OD) was measured with a plate reader (Titertek Multiskan MCC/340, Flow Laboratories, Allschwil Switzerland) at 570 nm (630 nm as reference). Cell viability was expressed as the percentage of the OD value of inhibitor-treated cells compared with untreated controls, according to the following equation: Viability = (OD SAMPLE/OD CONTROL) × 100. Untreated cells were used as control and each experiment was carried out in triplicate.

Cell cycle analysis

Cells cultured in 25 cm^2 flasks were trypsinized and washed with phosphate buffered saline (PBS). The cells suspensions in PBS (1×10^6 cells/ml) were centrifuged and the supernatant was removed. To quantify DNA content, cells were fixed in absolute ethanol and resuspended in 1 ml of hypotonic buffer containing 0.1% Triton X-100, 0.1% sodium citrate, and 50 mg/ml propidium iodide (Sigma Aldrich) in propylene FACS tubes. After centrifugation at 1100 × g for 5 min and supernatant removal, the cells were resuspended again in 250 μl of hypotonic buffer and incubated in the dark at 23 °C for 15 min. Finally, 250 μl of RNAse A solution (10 μg/ml RNAse A in PBS) (Sigma Aldrich) was added to each tube following by an incubation of 15 min in the dark at 23 °C. After this treatment and the addition of 0.5 ml of PBS, the cells were analyzed by flow cytometry (FACScan; Becton-Dickinson, Milan, Italy). Percentages of cells in G0/G1, G2/M phases were determined, using APC32 acquiring software and analyzed by APC32 analysis software on a Coulter EPICS cytometer.

Senescence-associated beta-galactosidase (SA-β-gal) activity assay

SA-β-gal activity was detected using in situ β-gal staining kit (Agilent Technologies, Cernusco sul Naviglio, Milan, Italy) according to the manufacturer's protocols. Briefly, attached cells were fixed in a buffer including 2% formaldehyde/0.2% glutaraldehyde for 10 min at 23 °C. After removing the fixing solution from the wells, the cells were washed twice with PBS pH 7.6 and incubated with a freshly prepared staining solution (pH 6) containing X-gal (5-bromo-4 chloro-3-indolyl- β-gal-actopyranoside) overnight at 37 °C in a humidified incubator. Finally, coverslips were mounted with Vectamount A (Vector Laboratories, Peterborough, UK). The percentage of blue-stained cells in the total number of cells was determined by counting cells with a Leica DM 5000B light microscope.

Real-time quantitative PCR (qRT-PCR)

The qRT-PCR technique was performed as previously described [29]. Briefly, total cellular RNA was isolated from both control and treated cell cultures, using TRIzol®REAGENT (Sigma-Aldrich) and according to the manufacturer's instructions. RNA (50 ng) was retrotranscribed using the ImProm-II Reverse Transciptase Kit (Promega Corporation, Milan, Italy) to obtain cDNA, which was amplified using the StepOnePlus™ Real-Time PCR System (Thermo Scientific, Milan, Italy). qRT-PCR analysis was performed using GoTaq qPCR Master Mix (A6001, Promega). The mRNA levels were normalized to the levels obtained for hypoxanthine phosphoribosyltransferase 1 (HPRT1), for betaglucuronidase (GUSB), and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cDNA was amplified using the primers indicated in Table 1. cDNA was amplified using the Rotor-gene™ 6000 Real-Time PCR Machine (Qiagen GmbH, Hilden, Germany). Changes in the transcript level were calculated using the $2^{-\Delta\Delta CT}$ method [30].

Western blotting

Treated and untreated (control) NCI-H292 cells were harvested with 0.25% trypsin supplemented with 1 mM EDTA (LONZA, Basel, Switzerland) and centrifuged at 1100× g for 5 min at 4 °C. Pellets were washed twice in PBS and resuspended in 100 μl ice cold RIPA lysis buffer (0.3 M NaCl, 0.1% SDS, 25 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton X-100, 0.5 mM DTT, 0.5% sodium deoxycholate) containing proteases and phosphatase inhibitors (0.1 mg/ml phenylmethyl sulfonyl fluoride, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 10 mg/ml NaF, 1 mM DTT, 1 mM sodium orthovanadate, 20 mM ß-glycerol phosphate) to obtain lysates. Cell lysates were incubated for 30 min on ice then centrifuged at 13,000× g for 20 min at 4 °C. The Bradford assay was used to determine the total protein concentration. Equal amounts (40 μg) of total cellular proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Segrate, Milan, Italy). Equal protein loading was ascertained by Ponceau-S staining of blotted membranes. After 1 h incubation at 23 °C with a blocking solution, 5% milk in Tris buffered saline pH 7.6 (TBS) with 0.05% Tween 20 (Sigma-Aldrich) (T-TBS), membranes were incubated with primary antibodies (mouse anti-Hsp60, LK1 clone, Sigma Aldrich; mouse anti-vimentin, V9 clone, Santa Cruz Biotechnology, Heidelberg, Germany; mouse anti-