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Evidence for the genotoxic effect of daunomycin in multipotent hematopoietic cells of mouse bone marrow: Chromatin proteins analysis

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ABSTRACT

Severe bone marrow suppression and anemia are the main side effects of using chemotherapeutic agents like daunomycin. In this study the effect of daunomycin on viability and chromosomal proteins of multipotent hematopoietic cells (MHCs) of mouse bone marrow was investigated. The cells were separated from mature cells according to their adherence, incubated in the absence and presence of various concentrations of daunomycin and viability was determined by trypan blue exclusion and MTT assay. The histones and high mobility group (HMG) proteins were extracted by acid and salt, respectively and analyzed on SDS-PAGE and immunoblot. The results revealed that daunomycin exhibited time and dose dependent cytotoxicity on the cells and upon increasing the concentrations of drug, the extractability of histone and HMG proteins decreased. Agarose gel electrophoresis showed aggregation of DNA in the presence of drug. Flow cytometry analysis suggested that there are differences in type and amount of histones H2A and H2B of these cells in comparison with thymus histones. Moreover histone H3 was not subjected to trimethylation at lysine 9 and daunomycin decreased H3K9 dimethylation and H3K9 acetylation in these cells in a dose-dependent manner. From these results it is concluded that the binding of daunomycin to chromatin precedes chromatin of multipotent hematopoietic cells of bone marrow into compaction/aggregation and beside DNA, histones and HMG proteins also play an important role in this process.

1. Introduction

Bone marrow is a tissue in which hematopoiesis occurs. Hematopoiesis is a complex process in which multipotent hematopoietic cells (MHCs) differentiate into special circulating erythroid, granulocyte, macrophage, megakaryocytic and lymphoid blood cells [1]. In vivo toxicity studies on hematopoietic cells, especially in terms of stem cell depletion, and direct in vitro exposure to chemotherapeutic agents like anthracyclines has showed a reduction in progenitor survival [2,3].

Daunomycin is one of the most active antitumor compounds widely used in clinical oncology, especially in the treatment of acute leukemias [4]. It mainly acts by intercalation of its planar aglycan chromophore between DNA base pairs, and its amino sugar ring lies in the minor groove of the double helix [5,6]. Although, large body of evidence introduces DNA as a main cellular target of daunomycin and its in vitro binding to DNA has been extensively studied in the past, there is no single mechanism of action responsible for all of the observed clinical and cellular effects of this drug.

As we know, in the cell nucleus, DNA exists as a complex structure called chromatin. It is associated with DNA binding proteins such as histones and nonhistone proteins building repeating units named "nucleosomes". These consist of 145 bp DNA wrapped around an octamer of basic proteins (core histones). There are five main histones in chromatin structure; a linker histone H1 and four core histones (H2A, H2B, H3 and H4) are arranged in an octamer form [7,8]. High mobility group (HMG) nonhistone chromosomal proteins are diverse, ubiquitous nuclear proteins which are existed in three subfamilies: HMGA, HMGB and HMGN [9]. HMGB subfamily is abundant and highly conserved nonhistone proteins that may exist in all eukaryotic cells nuclei [10]. Therefore the presence of these chromosomal proteins may affect and modulate the binding of daunomycin to DNA.

Abbreviations: BSA, bovine serum albumin; Gmean, geometric means; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffer saline; FITC, fluorescein isothiocyanate; TCA, trichloroacetic acid; DMEM, Dulbecco's Modified Eagle Medium; Ub, ubiquitin; H3K9me2, histone H3 lysine 9 dimethylation; H3K9Ac, histone H3 lysine 9 acetylation; MTT, 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide.

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As stated bone marrow is the main site of daunomycin cytotoxicity [2,11]. Although several attempts in this direction have already been carried out but the effect of daunomycin on cytotoxicity and chromatin of bone marrow cells are still poorly understood. In the present study, in order to elucidate the biological mechanism of daunomycin action, the effect of daunomycin on cytotoxicity and chromatin proteins of multipotent hematopoietic cells of mouse bone marrow cells was investigated. The results show that apart from lower content of H2A and H2B in these cells, daunomycin as an anticancer drug prevents extractability of histones and non-histone proteins via condensation of chromatin.

2. Materials and methods

2.1. Reagents

Daunomycin was purchased from Helale Ahmar Pharmacy (Tehran, Iran, manufactured by Pharmacia) and used without further purification. Stock solution of the drug was prepared in sterile distilled water at a concentration of 2 mg/ml and stored at −20 °C in the dark. Before use it was diluted to desired concentrations with 0.1 M PBS, pH = 7.4. Trypan blue, MTT, proteinase K, ECoRI-Hind III digested DNA marker, cocktail protease inhibitor, FITC or HRP-conjugated IgG were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). DMEM with 2 mM L-glutamine (Gibco, Invitrogen, Carlsbad, CA, USA) was supplemented with 3.7 g/l $NaHCO_3$, 15% heat-inactivated fetal calf serum (Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran) 30 mg/l asparagine, 100 U/ml penicillin and 10 μg/ml streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA).

Anti H3, H4, H2A, H2B, H3K9me2, H3K9me3, H3K9Ac and ubiquitin antibodies were purchased from Abcam (Cambridge, UK). Histones (H1 and core histones) and HMG proteins were prepared according to the procedures of Johns [12] and Goodwin et al. [13], respectively and used as a marker. Antibodies against H1, core histones and HMG proteins were prepared in IBB Laboratory (Tehran, Iran).

Male Balb/c mice weighting 20–25 g (6–8 weeks old) were obtained from the Laboratory Animal Center of IBB (Tehran, Iran). They were maintained in conventional pathogen free conditions in a temperature (22–23 °C), humidity (50–70%), and photoperiod (12 h dark/light cycle) controlled room.

2.2. Isolation of multipotent hematopoietic cells of bone marrow

Total bone marrow from each femur and tibia pair was eluted aseptically with 1 ml syringe and 25-gauge needle into 1 ml DMEM and aliquot of the cell suspension was diluted in 3% acetic acid to lyse red blood cells. The total nucleated cells were counted and the viability was determined by trypan blue exclusion assay. The bone marrow cells were cultured overnight in DMEM at the density of 10^6 cells per ml at 37 °C with 5% CO_2 and full humidified condition. Nonadherent cells which are the population of hematopoietic stem and progenitor cells were collected and used for further investigation [14].

2.3. Drug treatment and viability assay

Nonadherent multipotent hematopoietic cells of bone marrow (10^6 cells/ml) were cultured in the absence and presence of various concentrations of daunomycin for 4 h (time course study revealed that 4 h is a best incubation time). Daunomycin was added directly at the onset of the culture and the cultures without daunomycin were used as control. Viability of the cells was determined as is described below.

Trypan blue: Drug treated and the control was mixed with an isotonic solution of trypan blue (0.4%, w/v) and viability of the cells was determined by trypan blue exclusion assay.

MTT assay: The method of Mossman [15] was used with some modifications. The cells (8×10^4 cells/well) were seeded into a 96-well plate (Nunclon, Denmark) and incubated with a series of drug concentrations for 4 h (to control wells only culture medium was added). Then 10 μl of MTT (5 mg/ml in H_2O) was added to each well and the cells were incubated at 37 °C with 5% CO_2 for 4 h. Then the medium was aspirated and replaced with 100 μl DMSO per well in order to dissolve the formed violet formazan crystals within the metabolically viable cells. The plates were gently shaken for 5 min at room temperature to allow complete dissolving of the formazan and the absorbance was read at 570 nm with a BioTek ELISA microplate reader (Model Power Wave XS2, Bio Tek, USA). The relative viability of the treated cells was expressed as the percent of the control.

2.4. Extraction of histones, HMG proteins and DNA

Histones and nonhistone proteins extraction: Multipotent hematopoietic cells of bone marrow (2×10^6) were incubated in the absence and presence of various concentrations of daunomycin as described above. Total histones (all five histones) and ubiquitin were isolated from the cells with 0.3 N HCl as described by Johns [12]. High mobility group (HMG) proteins were isolated with 0.35 M NaCl as described by Goodwin et al. [13]. After precipitation with 12% TCA, the proteins were dissolved in the SDS gel loading buffer for polyacrylamide gel electrophoresis.

DNA extraction: Drug treated and the controls were washed twice with ice-cold PBS and resuspended in DNA lysis buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS and 50 μg/ml proteinase K), followed by incubation at 56 °C for 4 h. Subsequently, equal volume of phenol was added to each solution, and the aqueous phase was recovered using a mixture of chloroform–isoamyl alcohol (24:1). Sodium acetate (3 M) (1/10 volume) and absolute ethanol (2 volumes) were added and incubated at −20 °C for 2 h. The samples were then eppendorfed for 5 min at 4 °C, washed with ethanol and air dried. DNAs were resuspended in TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA) and DNA sample solvent, heated at 65 °C and electrophoresed on agarose gel.

2.5. Gel electrophoresis and western blot

SDS-PAGE: The protein samples were analyzed on 15% SDS polyacrylamide gel electrophoresis as described by Laemmli [16]. The samples (100 μl) were directly equilibrated with SDS sample buffer or precipitated with 12% TCA and then after added sample buffer loaded. The gel was run for 1.5 h at 100 V, stained with 0.1% Coomassie brilliant blue R 250, destained in 10% methanol/acetic acid and photographed.

Western blot: The protein samples were run on 15% polyacrylamide gel at 100 V for protein separation. The proteins were then transferred onto a nitrocellulose membrane [17]. The membrane with the immobilized protein bands was incubated for 1 h at 37 °C with 1% (w/v) gelatin in Tris–NaCl buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl), referred to as blocking buffer, and washed three times of 5 min with Tris–NaCl buffer. The membrane was then incubated overnight with overall anti rabbit specific antibodies (e.g. total histones and HMG proteins) or anti rabbit specific anti histones (H2A, H2B, H3, H4, Ub) individually at 4 °C. After three times washing the membrane with Tris–NaCl/Tween somehow 20 (0.05%), it was incubated with peroxidase-conjugated goat anti rabbit IgG for 2 h at room temperature. After washing (three times), the membrane was incubated with the substrate solution (1.2 ml of 0.3% 4-chloro-1-naphtol in methanol was mixed with 20 ml