Mutation Research 789 (2016) 9–14

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Short communication

Different repair kinetic of DSBs induced by mitomycin C in peripheral lymphocytes of obese and normal weight adolescents

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ARTICLE INFO

Article history:
Received 4 August 2015
Received in revised form 21 March 2016
Accepted 1 May 2016
Available online 3 May 2016

Keywords:
γ-H2AX
DSBs
Peripheral lymphocytes
Obesity
HMGB1

ABSTRACT

In 2013, 42 million children under the age of 5 years were overweight or obese. In the context of obesity, we recently showed that (1) peripheral lymphocytes of obese children/adolescents had an 8-fold increase in double strand breaks (DSBs), expressed as g-H2AX foci, than normal weight adolescents, and (2) 30% of the damage was retained into chromosome mutations. Thus, we investigated DSBs repair efficiency in a group of obese adolescents assessing the kinetic of H2AX phosphorylation in mitomycin C (MMC)-treated lymphocytes harvested 2 h- or 4 h-post mutagen treatment. According to our previous studies, these harvesting times represent the peak of DSBs induction and the time in which an appreciable DSBs reduction was observed. In addition, we evaluated the expression of the high mobility group box-1 protein (HMGB1), a chromatin remodelling protein involved in DSBs repair and obesity. Compared to normal weight adolescents, obese subjects 1) showed higher levels of g-H2AX foci at either 2 h- (0.239 ± 0.041 *vs.* 0.473 ± 0.048, P = 0.0016) or 4 h- (0.150 ± 0.026 *vs.* 0.255 ± 0.030, P = 0.0198) post mutagen treatment, and 2) have repaired a greater amount of the initial lesions (0.088 ± 0.033 *vs.* 0.218 ± 0.045, P = 0.0408). Concordantly, 1) HMGB1 levels of obese individuals increased and decreased at 2h- or 4 h-post mutagen treatment, respectively, and 2) the opposite occurred for the normal weight adolescents where the protein was down-expressed at 2 h and over-expressed at 4 h. In conclusion, lymphocytes of obese and normal weight adolescents showed a distinct temporal kinetic of repairing MMC-induced DSBs, together with a different expression of HMGB1. The finding that obesity may modulate the repair of DNA damage induced in lymphocytes by genotoxic agents should be confirmed by further experiments.

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1. Introduction

The worldwide prevalence of obesity is more than doubled between 1980 and 2014. In 2013, 42 million children under the age of 5 were overweight or obese [1]. Obesity is characterized by chronic low-grade inflammation, a series of metabolic changes at the level of the adipose tissue that consist in decreased or increased production of anti-inflammatory molecules or pro-inflammatory cytokines, respectively, hypoxia and oxidative stress [2–6]. The adipose tissue is in fact considered a relevant source of free radicals and ROS, since the redox regulation plays a pivotal role in the adipogenesis [7,8]. The increase of free radicals in the accumulated fat can induce, not only in the target tissue but also in other districts, DNA lesions such as the double-strand breaks (DSBs) that in turn can initiate and promote carcinogenesis. These lesions can be easily detected in a cell population as nuclear γ-H2AX foci shortly after their induction (within a few minutes) [9], and the *ad hoc* developed assay has now become the most widely used method for investigating the presence of early genome damage in the form of DSBs [10–14]. Following a DSBs, modifications to chromatin conformation are necessary; specifically, chromatin should be relaxed to allow and facilitate the access to the repair machinery in the region of the lesion [15,16]. The dephosphorylation of γ-H2AX indicates the end of the DDR response, *i.e.* that the DSBs have been correctly repaired. Thus, evaluating the kinetic of appearance and disappearance of γ-H2AX foci allows us to investigate the efficiency of the DSBs repair in a given cell population. In addition, the multifunctional high mobility group box-1 protein (HMGB1) was reported to have a key role in DSBs repair participating in the chromatin

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http://dx.doi.org/10.1016/j.mrfmmm.2016.05.001
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Table 1
Demographic and clinical characteristics of the study population. Data are expressed as mean ± SEM.

	Normal weight	Obese	Total
Subject (n)	15	23	38
Female% (n)	53.33 (8)	52.17 (12)	52.63 (25)
Age (years)	11.07 ± 0.83	12.44 ± 0.42	11.76 ± 0.4
BMIZ-score	0.69 ± 0.13	2.84 ± 0.13	1.99 ± 0.19

remodelling at the sites of induced DNA damage [17]. Recent studies have also shown that in response to inflammatory signals, the adipose tissue, in particular adipose-derived stromal cells, of obese adults, was able to produce levels of HMGB1 2-fold higher than those of normal weight subjects [18], and that children had an increase in plasma levels of this protein [19]. In addition, HMGB1 has been reported to be involved in contributing and maintaining a low-grade inflammation in human preadipocytes [20].

With regard to obesity, we have recently reported that (1) peripheral lymphocytes of obese children/adolescents had an eight-fold increase in DSBs as compared to a normal weight group, and (2) a part (about 30%) of this initial genome damage was retained into chromosome mutations [21]. In another work by us we set up the proper experimental conditions to study the time-course of H2AX phosphorylation in human lymphocytes treated *in vitro* with physical or chemical mutagens [22]. Thus, in the light of the above mentioned study carried out on the close association between obesity and DNA damage, the aim of the present work was to investigate whether or not obese adolescents have a different efficiency in repairing the DSBs lesions than normal weight adolescents. To do this, using the γ-H2AX assay, we assessed the kinetic of repair of DSBs induced by mitomycin C (MMC) in peripheral lymphocytes after two and four hours of mutagen treatment. In addition, we aimed to assess, by western blotting, the levels of the HMGB1 protein and its variation under the same experimental conditions.

2. Materials and methods

2.1. Study populations

The study populations consisted of 38 adolescents (15 normal weight and 23 obese) recruited at the Unit of Pediatric Endocrinology and Diabetes, Department of Clinical and Experimental Medicine, Azienda Ospedaliero-Universitaria Pisana (Pisa, Italy). Only subjects who had not received radiation exposure or drug therapy, free from any pathological conditions, non-smokers and non-alcohol drinkers, were included in the study. Obese adolescents were recruited for the collection of blood during their specialist visit. Controls were selected from subjects who needed to have a blood test examination, *e.g.* in the case of familiar short stature or of planned minor surgery, or who had voluntarily accepted to take part in the study. Adolescents were defined as normal weight or obese on the basis of their body mass index (BMI) Z score, using the International Obesity Task Force BMI cut-offs as a reference [23]. BMI Z scores were then calculated using the least mean square method, reference values of BMI, and the least mean square coefficients [24]. Informed consent was obtained from the parents, or from their legal representatives, before adolescents took part in the study. The protocol was approved by the Azienda Ospedaliero-Universitaria Pisana ethical committee. Table 1 shows the demographic and clinical characteristics of the study population.

2.2. Cell cultures, mitomycin C treatment and cell harvesting

6 ml of blood in lithium heparin tubes were collected by venipuncture from all subjects and cultured on the same day. Culture tube containing 150 μl of whole blood and 2.35 ml of RPMI-1640 medium (Invitrogen, Milano, Italy) was supplemented with 20% FBS (Invitrogen), 1% antibiotic/antimycotic (Invitrogen) and 1.5% phytohaemagglutinin (PHA; Invitrogen). The cells were incubated at 37 °C for 20 h. We treated the stimulated T-lymphocytes *in vitro* with a single dose of a chemical mutagen, the anticancer drug mitomycin C (MMC; Sigma-Aldrich, Milano, Italy), (final concentration: 0.3 μg/mL). The chemical mutagen was dissolved in sterile H_2O and the corresponding negative control culturesreceived only the solvent used. Treated or untreated cells were then transferred to culture tubes at room temperature and harvested after 2 and 4 h. Cell harvesting was carried out according to the procedure described elsewhere [22]. Briefly, after hypotonic treatment with 0.075 M KCl, lymphocytes were pre-fixed in acetic acid:methanol 5:3, fixed in 100% methanol, washed twice in methanol:acetic acid (3:1) and dropped onto a clean glass slide. The air-dried slides were immediately processed by the immunofluorescence protocol for visualisation of γ-H2AX nuclear foci. For each subject we set up a series of at least two independent cultures per experimental point.

2.3. Immunofluorescence for γ-**H2AX analysis**

For the detection of γ-H2AX we used a phospho-histone H2AX (Ser-139) polyclonal primary antibody (Cell Signaling, Euroclone, Milan, Italy) and the DyLight 488-conjugated anti-rabbit secondary antibody (Pierce, Euroclone, Milan, Italy). Cells were washed twice in 1X phosphate-buffered saline (PBS) blocked in 1X PBS/10% FBS/0.3% Triton X-100 (Sigma Aldrich, Milan, Italy) as permeabilization or blocking solution (PS), cells were blocked in PS for 30 min at room temperature and incubated overnight at 4 °C with primary antibody diluted 1:50 in PS. The following day, cells were washed 3 times in 1X PBS, incubated at room temperature for 2 h with secondary antibody diluted 1:200 in PS, and, after washing three times in 1X PBS, slides were counterstained with 0.4 μg/mL of 4′,6′-diamidino-2-phenylindole (DAPI) in antifade solution (Fluoroguard, LiStarFish, Milano, Italy). Slides were then analyzed on a Nikon-Optiphot 2 fluorescent microscope properly equipped with filters for DAPI and FITC visualisation using a x100 lens (x1000 final view). The presence of γ-H2AX focus was detected as a green fluorescence spot in the blue counterstained nucleus. For each experimental point, at least 400 cells per culture were scored and the DNA damage induced by MMC is expressed as average number of γ-H2AX foci per nucleus (γ-H2AXF/N) obtained from two independent replicates. To quantify the kinetic of repair, we measured in both obese and normal weight adolescents DNA repair. The number of γ-H2AX foci per nucleus in MMC-treated and untreated cultures at 2 h or 4 h gave the actual level of induced or unrepaired DNA damage, respectively. The difference between these parameters allowed estimating the level of damage which has been repaired from 2 to 4 h (repaired DNA damage).

2.4. Immunoblotting analysis

An aliquot of heparinised whole blood from each adolescent was employed to separate white cells using the following protocol. 3 ml of whole blood and 3 ml of sterile 1X PBS (1: 1 dilution) were mixed in a sterile tube. Then, 3 ml of the mix were gently added to another tube containing 3 ml of Histopaque-1077 (Sigma-Aldrich, Milan, Italy). The mononuclear white cell ring, obtained by a centrifugation at 2100 rpm for 30 min, was carefully collected, washed twice in sterile PBS and resuspended in the appropriate amount of PBS to

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