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Methylglyoxal Induces Mitochondria-Dependent Apoptosis in Sarcoma

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Abstract—In the preceding paper (A. Ghosh et al. (2011) *Biochemistry* (Moscow), **76**, 1051-1060), using several comparable tissue materials, it has been convincingly demonstrated that methylglyoxal, a normal metabolite, inhibits mitochondrial complex I of specifically malignant cells. This suggests a distinct alteration of complex I, a highly important enzyme for energy (ATP) production, in malignancy. The present paper shows that as a consequence of this inhibition mitochondrial membrane potential is drastically reduced in sarcoma tissue but not in normal skeletal muscle. This was estimated spectrofluorimetrically using the dye rhodamine 123. As a consequence, cytochrome *c* was released from the sarcoma mitochondria as evidenced by Western blot analysis. Moreover, on treatment with methylglyoxal membrane potential collapse of sarcoma 180 cells was also indicated by fluorescence-activated cell sorter analysis. Atomic force microscopic study demonstrated gross structural alteration specifically of tumor mitochondria on methylglyoxal treatment. All these studies suggest that methylglyoxal might initiate an apoptotic event in malignant cells.

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Key words: sarcoma, methylglyoxal, membrane potential, atomic force microscopy, cytochrome *c*

As early as 1913 it was observed that methylglyoxal was degraded to lactic acid by the ubiquitous glyoxalase enzyme system. However, the precise enzymatic formation and breakdown of this compound have been elucidated only recently. Similarly, the first report of the anticancer effect of methylglyoxal was in 1958 (reviewed in [1-3]). In the 1960s Szent-Gyorgyi and his collaborators championed the idea that it might regulate cellular growth and could serve as a potent anticancer agent [4], but the mechanism of the anticancer effect was deciphered only recently. It had been observed in our laboratory that methylglyoxal inhibits both glycolysis and mitochondrial respiration, thereby depleting energy production specifically of malignant cells. Further experimental evidences indicated mitochondrial complex I of specifically malignant cells is the target of methylglyoxal [1, 3].

In the preceding paper [5] we further deciphered this phenomenon in a more defined way by using mitochondria from normal muscle and sarcoma tissue. We used respiratory studies with intact mitochondria and submitochondrial particles as well as spectrophotometric assay of NADH dehydrogenase (mitochondrial complex I). Moreover, the work was extended with a wide variety of human normal and malignant tissues, which convincingly demonstrated that the effect of methylglyoxal is on mitochondrial complex I of exclusively malignant tissues. All these results strongly suggest that mitochondrial complex I might be critically altered in all malignant cells [5].

In the present paper we investigated the consequences of inhibition of mitochondrial complex I of malignant cells by methylglyoxal. Reduction in respiratory complex I activity leads to decrease in membrane potential, which is an early step in apoptosis [6, 7]. The decrease in membrane potential across the mitochondrial inner membrane results in efflux of cytochrome *c* from the mitochondrial matrix to cytosol. So we investigated and compared in normal and malignant cell mitochondria: i) the status of membrane potential spectrofluorimetrically and by FACS (fluorescence-activated cell sorter) analysis using dyes rhodamine 123 and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide (JC-1), respectively; ii) release of cytochrome

Abbreviations: AFM, atomic force microscopy; FACS, fluorescence-activated cell sorter; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide; 3MC, 3-methyl cholanthrene.

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c by Western blot analysis, and iii) structural alteration by atomic force microscopy. In consonance with the results described in the preceding paper, here also we observe that methylglyoxal is detrimental specifically against malignant cell mitochondria.

MATERIALS AND METHODS

Chemicals. 3-Methyl cholanthrene (3MC), rhodamine 123, JC-1, methylglyoxal, protease VIII (product No. P5380), rotenone, α-oxoglutarate, ADP (disodium salt), succinate, and malonate were obtained from Sigma Chemical Co. (USA). Muscovite mica pieces were obtained from ICR & Sons Pvt. Ltd. (India). All other reagents used were of analytical grade and purchased from local manufacturers.

Development of sarcoma in mice. Animal experiments were carried out in accordance with the guidelines of Institutional Ethics Committee (IEC). Appropriate precautions were taken to minimize pain and discomfort to animals. Sarcoma tissue was developed as described previously by injecting 3MC at the dose of 10 mg/kg body weight in the hind leg of Swiss albino female mice. Malignancy was confirmed by histological examination [8]. Sarcoma tissues were excised soon after sacrificing the mice and immediately transferred to ice cold buffer.

Cell line. Sarcoma 180 cells were maintained in the peritoneal cavity of Swiss albino female mice by weekly inoculation of $2 \cdot 10^6$ cells.

Preparation of mitochondria from different sources. Unless mentioned otherwise, all operations were carried out at 0-4°C. Mouse skeletal muscle and sarcoma tissue mitochondria were prepared as described in the preceding paper [5].

Sarcoma 180 cells. Mitochondria from sarcoma 180 cells were prepared essentially by digitonin permeabilization by the method of Moreadith and Fiskum with some minor modifications [9]. Sarcoma 180 cells were collected and washed in normal saline by centrifugation at 180*g* for 5 min. Then the pellet was resuspended in H-medium (+EGTA) containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 0.02% BSA (fatty acid free), and 1 mM EGTA, pH finally adjusted to 7.2 with dilute KOH. The suspension was centrifuged at 625*g* for 5 min, and the pellet was collected. The pellet was then resuspended in H-medium (+EGTA) to a cell density of $(1\text{-}2) \cdot 10^8$ cells/ml. Digitonin solution was added to it in small aliquots until 90-95% of the cells became non-viable. Then the volume was doubled by adding H-medium (+EGTA) and centrifuged at 3000*g* for 10 min. The gelatinous pellet was resuspended to 50% of the previous volume and homogenized in a tight fitting Potter–Elvehjem homogenizer with 12 up and down strokes. Its volume was then increased 3 times with H-medium (+EGTA) and centrifuged at 1000*g* for 15 min. The supernatant was collected and centrifuged at 12,000*g* for 15 min. The pellet was resuspended to 50% of the previous volume in H-medium (–EGTA), which had the same composition like that of H-medium (+EGTA) with the exception that EGTA was not present. Then it was centrifuged at 10,000*g* for 15 min, and the pellet was collected. Finally the tightly-packed pellet was suspended in a minimum volume of H-medium (–EGTA), and this mitochondrial suspension was used for respiratory studies.

Measurement of mitochondrial respiration. Mitochondrial oxygen consumption was measured with a Hansatech Oxygraph (GB) fitted with a Clark electrode as described in the preceding paper [5].

Membrane potential assay. *Spectrofluorimetric measurement of membrane potential using rhodamine 123.* Changes of mitochondrial membrane potential were measured by using rhodamine 123 in a spectrofluorimeter with different respiratory substrates and inhibitors of oxidative phosphorylation [10]. Highly coupled mitochondria were prepared as mentioned above. The respiratory control ratio for all types of mitochondria was usually >5. Rhodamine 123 was dissolved in ethanol, and its concentration was estimated spectrophotometrically at 507 nm (ε_{507} = 101 $mM^{-1} \cdot cm^{-1}$). Mitochondrial membrane potential changes were monitored by measuring rhodamine 123 fluorescence quenching by exciting the fluorochrome at 503 nm and detecting the fluorescence emission at 527 nm. Mitochondria containing 0.35-0.40 mg of protein were added to 2 ml of reaction medium containing 250 mM sucrose, 10 mM HEPES, pH 7.4, 0.1 mM EGTA, 2 mM $MgCl_2$, 4 mM KH_2PO_4, and 50 nM rhodamine 123. Other additions are mentioned in Fig. 1 (see further). ADP or ADP regenerating system (10 mM glucose, 2 U hexokinase, and 0.4 mM ATP) was added to start the phosphorylation reaction. During the measurements the reaction medium was continuously stirred at 30°C.

FACS analysis of sarcoma 180 cells. Sarcoma 180 cells from peritoneal exudates were harvested by sterile and chilled 50 mM PBS (phosphate buffered saline) and then cultured in DMEM + 10% FBS (10^6 cells/ml) at 37°C in a humidified atmosphere of 5% CO_2. After 4-6 h, adhering cells were removed, and sarcoma 180 (non adherent) cells were incubated with or without methylglyoxal for 12 h in fresh medium. After incubation, the sarcoma 180 cells were washed in PBS and stained with 25 μM JC-1 (a potentiometric probe for mitochondria) for 30 min at 37°C in the dark. The cells (10^4 cells) were then analyzed to determine mitochondrial membrane depolarization using a flow cytometer (Calibur; BD Biosciences) and CellQuest software [11].

Western blot analysis. Mitochondria containing approximately 1 mg protein were suspended in 1 ml of incubation medium containing 250 mM sucrose, 10 mM HEPES, pH 7.4, 0.1 mM EGTA, 2 mM $MgCl_2$, and 4 mM KH_2PO_4 with 10 mM pyruvate plus 10 mM malate

and 0.4 mM ADP. This suspension was incubated with or without methylglyoxal for 30 min at 37°C. After incubation, the supernatant was concentrated 20-fold using Centricon and Millicon membrane filters (Millipore; 10 kDa). Concentrated supernatants were used for Western blot analysis. Protein was transblotted using a Mini Transblotter (Bio-Rad, USA). Primary antibody (diluted 1 : 200) against cytochrome *c* and secondary antibody (diluted 1 : 500) of anti-mouse HRP-conjugated (IgG2b) were used. Immunoreactive bands were visualized using chemiluminescence Western blotting kit (Santa Cruz Biotechnology, USA).

Atomic force microscopy (AFM) experiment. AFM images were taken according to Layton et al. with minor modification [12]. Mouse skeletal muscle and sarcoma tissue mitochondria were prepared as described in the preceding paper [5]. Final mitochondrial pellets were suspended in buffer containing 0.1 M KCl, 0.05 M Tris-HCl, pH 7.4, 2 mM EGTA at about 100 mitochondria per µl. Two samples, one from normal and one from sarcoma tissue, were challenged with 2.5 mM methylglyoxal and kept for 1 h at 37°C; two other samples were kept similarly without methylglyoxal. After incubation, 4 µl of mitochondrial suspension were deposited on freshly cleaved Muscovite mica pieces and kept for 1 h. These samples were then washed quickly with 5 ml (5 × 1 ml) of MilliQ water using a micropipette and dried with a gentle stream of nitrogen gas and imaged immediately.

During the washing of mitochondrial samples adsorbed on mica surface, a very quick water flow from a

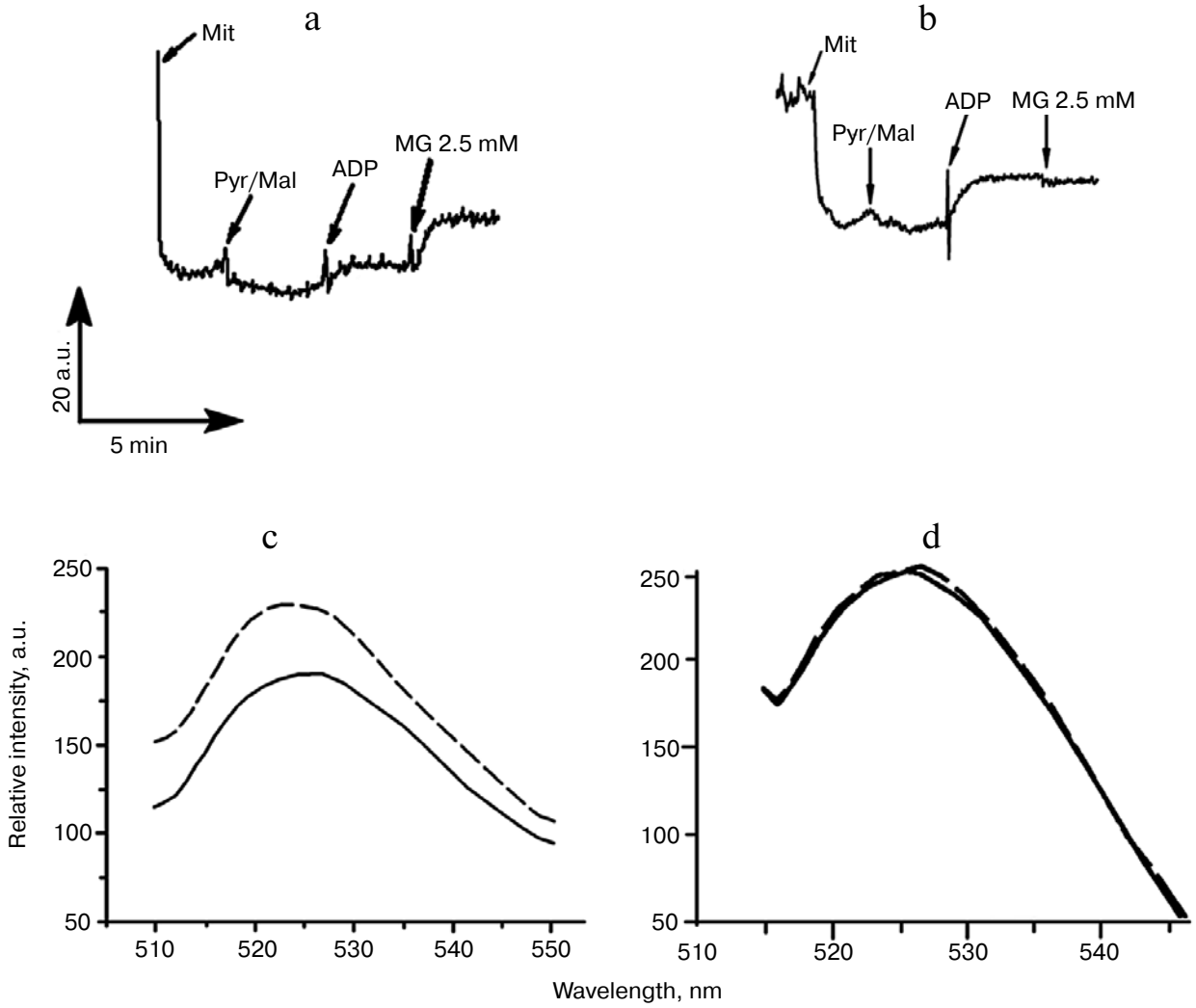

Fig. 1. Mitochondrial membrane potential assay of sarcoma tissue (a, c) and skeletal muscle (b, d) mitochondria by rhodamine 123. a, b) Time course of rhodamine 123 fluorescence when incubated in presence of sarcoma (a) and skeletal muscle (b) mitochondria, upon addition of respiratory substrates and methylglyoxal. Additions, where indicated, were mitochondria (Mit), 10 mM pyruvate + 10 mM malate (Pyr/Mal), 0.4 mM ADP (ADP), and 2.5 mM methylglyoxal (MG). c, d) Fluorescence spectra of rhodamine 123 when incubated in the presence of sarcoma tissue (c) and skeletal muscle (d) mitochondria (0.2 mg protein/ml), respectively, either alone (solid line) or in the presence (dotted line) of methylglyoxal (2.5 mM) without any other addition.

micropipette was used to remove unwanted buffer materials. This is because during the imaging these materials can damage the AFM tips and thus hinder the imaging process. Once a mitochondrion adsorbed on the mica, it was fixed on the two dimensional surface and could not rotate in three dimensional space. In such case it is very unusual if water causes osmotic imbalance and rupture of mitochondria.

Data acquisition and analysis. All the images of mitochondrial preparations were carried out using a Pico LE model of Agilent Corp. (USA) in an ambient condition of 25 ± 2°C. Imaging was performed in the intermittent contact mode using Acoustic Alternative Current (AAC) for the oscillatory motion of the cantilever (μmasch). The cantilever used had frequencies within 170-325 kHz and force constant value 4.5-27.5 N/m. Scan speed was 0.8-1.3 lines/sec. All data presented in this study were verified by sampling at least three different areas and confirmed in three sets of animals. All images presented here (see further Fig. 3) are topographic and are raw data except for minimum processing limited to third order flattening. The dimension of the mitochondria was estimated along the long axis of the near spherical samples. The height values (see further Fig. 4) were measured as the difference between the highest points in the cross-sectional diagram and the average height of the mitochondrial surface. The width values were measured as full width at half maxima of the cross-sectional diagram.

Protein was estimated with BSA as standard by the method of Lowry et al. as described by Layne [13].

Statistical analysis. Statistical analysis was performed using Origin 6 software. Each experiment was performed 3 to 5 times and results are expressed as mean ± SD; Student's *t*-test for significance was performed and $p < 0.05$ was considered significant. Western blot analysis data show representative data of at least three independent experiments.

RESULTS AND DISCUSSION

Assay of membrane potential change (Δψ) of skeletal muscle and sarcoma tissue mitochondria. In the preceding paper we showed that methylglyoxal inhibits the mitochondrial respiration of 3MC-induced fibrosarcoma and a wide variety of human adenocarcinoma and carcinoma, but it had no significant effect on mitochondrial respiration of mouse skeletal muscle and other human normal tissue such as liver, stomach, colon, gallbladder, etc. [5]. It appears that this specificity of methylglyoxal against the NADH dehydrogenase (complex I) of malignant tissue mitochondria is one very important reason for its selective anticancer property. Reduction in respiratory complex I activity leads to decrease in membrane potential, which is an early step in apoptosis [6, 7]. So we monitored spectrofluorimetrically the effect of methylglyoxal on mitochondrial membrane potential of both sarcoma and skeletal muscle by using the dye rhodamine 123.

Figure 1 shows that at a concentration of 2.5 mM, methylglyoxal was found to increase the fluorescence by dissipating mitochondrial membrane potential of sarcoma tissue (a) but not of normal skeletal muscle (b). Similarly, the emission spectrum of rhodamine 123 further demonstrated that in the absence of any respiratory substrate and inhibitor, methylglyoxal at a concentration of 2.5 mM induces decrease in membrane potential in sarcoma mitochondria only (c) but not in skeletal muscle mitochondria (d). Our finding corroborates an earlier report showing that methylglyoxal induces apoptosis through reduction in mitochondrial membrane potential followed by caspase 3 activation in Jurkat leukemia T cells [14].

Release of cytochrome *c* from sarcoma tissue mitochondria. It is also well established through numerous *in vitro* and *in vivo* studies that mitochondrial proapoptotic factor cytochrome *c* is released due to loss of membrane potential and subsequent opening of the permeability transition pore, initiating an apoptotic signaling cascade [15, 16]. To understand the mode of cell death induced by methylglyoxal, cytochrome *c* was assayed by Western blotting technique.

When mitochondria from skeletal muscle and sarcoma tissue were incubated in the presence of 2.5 mM methylglyoxal for 30 min at 37°C, a significant amount of cytochrome *c* was released only from the intact sarcoma mitochondria to the incubation medium (Fig. 2). Release of cytochrome *c* specifically in sarcoma mitochondria is significantly reduced by cyclosporine A, a known blocker of mitochondrial permeability transition pore through which cytochrome *c* is released into the extramitochondrial space [17]. No release of cytochrome *c* was observed in

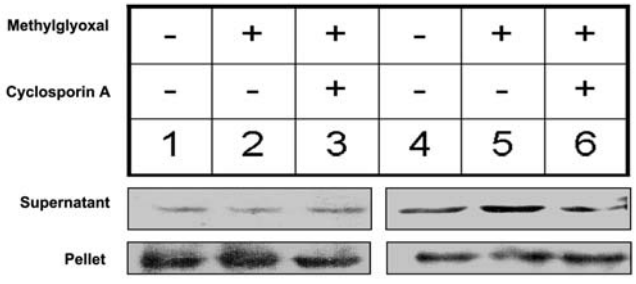

Fig. 2. Release of cytochrome *c* from sarcoma mitochondria induced by methylglyoxal. Mitochondria of skeletal muscle (lanes *1-3*) or sarcoma (lanes *4-6*) containing 1 mg/ml protein were incubated at 37°C for 30 min either without (–) any addition or in presence (+) of 2.5 mM methylglyoxal or in presence of 2.5 mM methylglyoxal plus 33 nM of cyclosporine A. Samples were then centrifuged at 10,000*g* for 10 min at 4°C to separate mitochondria and supernatant. Both the supernatant and the pellet were subjected to 15% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with monoclonal anti-cytochrome *c* antibody.

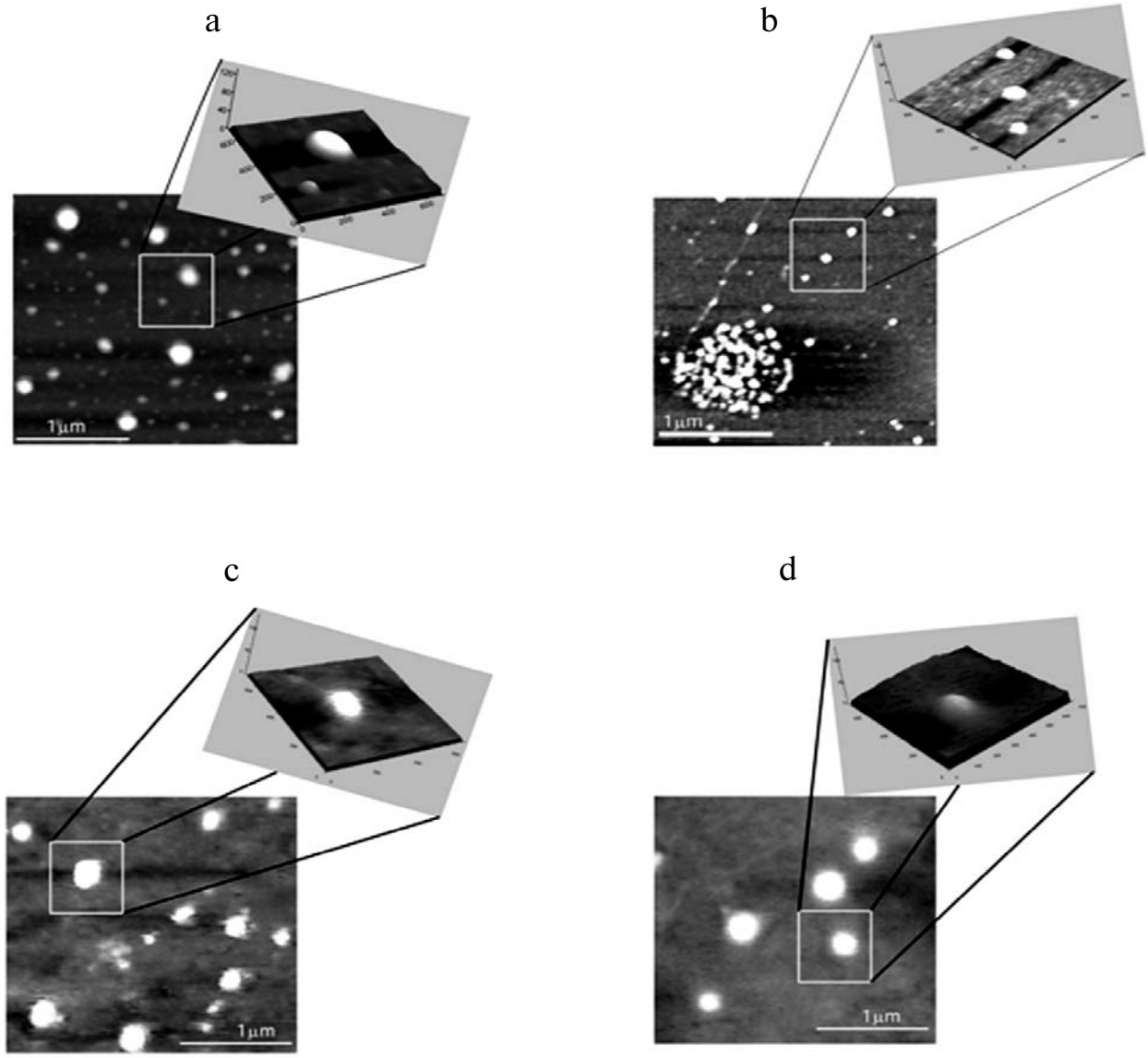

Fig. 3. Atomic force microscopic images of sarcoma (a, b) and skeletal muscle (c, d) mitochondria in presence (b, d) and in absence (a, c) of methylglyoxal (2.5 mM). Insets show a larger 3D configuration of mitochondria.

skeletal muscle mitochondria, which further corroborated with the data of Speer et al. that methylglyoxal suppresses the permeability transition pore in normal rat liver mitochondria [18].

Structural difference between mouse skeletal muscle and sarcoma tissue mitochondria with and without methylglyoxal as evidenced by atomic force microscopy. The structural changes of sarcoma and skeletal muscle mitochondria after treatment with methylglyoxal were investigated using AFM. Previously AFM topographs were successfully used to obtain direct three-dimensional conformational changes of mitochondria after drug treatment [12]. These types of studies are generally performed with electron microscopy in a fixed section. There are several advantages of AFM over electron microscopy: i) it can be studied without labeling or staining, and ii) data are relatively easy to interpret in comparison with other techniques [19].

Approximately 240 mitochondria were analyzed from a total four sets of samples. These images are presented in Fig. 3, which shows overall surface topology of mitochondria. Both methylglyoxal treated and untreated groups of mitochondria displayed similar clustering configuration. We also measured approximate size of mitochondria. Sarcoma mitochondria had an average width of 161 ± 23 nm and a height of 8 ± 1.4 nm, whereas skeletal muscle mitochondria had average width and height of 120 ± 7 and 6 ± 2.5 nm, respectively. Sarcoma mitochondria, when challenged with 2.5 mM methylglyoxal for 1 h, had undergone a massive change in size. Mitochondrial width and height were significantly reduced to 93 ± 6 and 6.4 ± 1.5 nm, respectively.

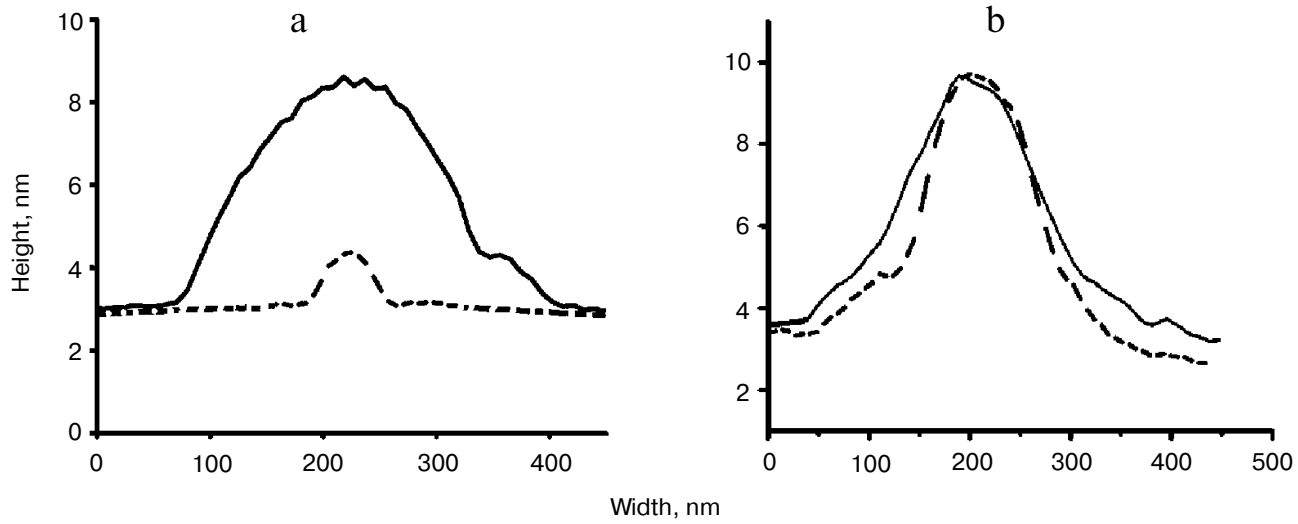

Fig. 4. Cross sectional analysis of a typical AFM image of sarcoma (a) and skeletal muscle (b) mitochondria without (solid line) and with 2.5 mM methylglyoxal (dashed line).

Muscle mitochondria challenged with methylglyoxal showed no significant change in mitochondrial dimension (width 130 ± 22 nm and height 6.6 ± 1.9 nm).

Figure 4 represents cross sectional analysis of both methylglyoxal treated and untreated sarcoma (a) and skeletal muscle (b) mitochondria. Methylglyoxal decreased membrane potential, which in turn triggered the opening of the permeability transition pore (PTP). Opening of the PTP usually results in mitochondrial swelling, rupture of the outer membrane, and cytochrome *c* release. But in sarcoma mitochondria methylglyoxal induced mitochondrial shrinkage. This might be due to rupture of mitochondrial membrane and consequent escape of mitochondrial contents in the medium. But the detail mechanism could not be resolved at this stage.

Effect of methylglyoxal on respiration and membrane potential of sarcoma 180 cell mitochondria. For these experiments we used sarcoma 180 cells, a model malignant cell. Figure 5 shows the effect of methylglyoxal on mitochondrial respiration of sarcoma 180 cells with different respiratory substrates and inhibitors. Methylglyoxal at a concentration of 2.5 mM inhibits mitochondrial α-oxoglutarate (α-OG)-dependent ADP-stimulated respiration of sarcoma 180 cells to the extent of 88%, which could be recovered by 5 mM succinate (complex II specific respiratory substrate) and further inhibited by 0.5 mM malonate. As expected, rotenone (5 μM), a well-known inhibitor of mitochondrial complex I, also completely inhibited α-OG-dependent ADP-stimulated respiration (data not shown).

Dissipation of membrane potential was further detected by FACS analysis of methylglyoxal-treated intact sarcoma 180 cells. As described in "Materials and Methods", after incubation with methylglyoxal for 12 h, sarcoma 180 cells were washed with PBS and stained with 25 μM JC-1. A significant dose-dependent change of red to green fluorescence was observed (Fig. 6), which implied mitochondrial transmembrane depolarization. Methylglyoxal at a concentration 1 mM showed maximum depolarization, and increasing the concentration of methylglyoxal did not enhance the membrane depolarization any more. This was corroborated with the change

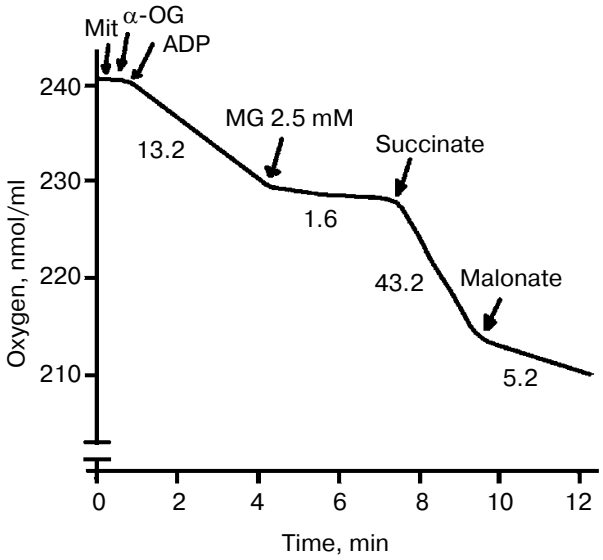

Fig. 5. Effect of methylglyoxal on mitochondrial respiration of sarcoma 180 cells. Ordinate, oxygen consumption by mitochondria of sarcoma 180 cells. The values along the Oxygraph tracing represent oxygen consumption rate in nmol O_2/min per mg protein. Mit, mitochondria; MG, methylglyoxal; α-OG, α-oxoglutarate.

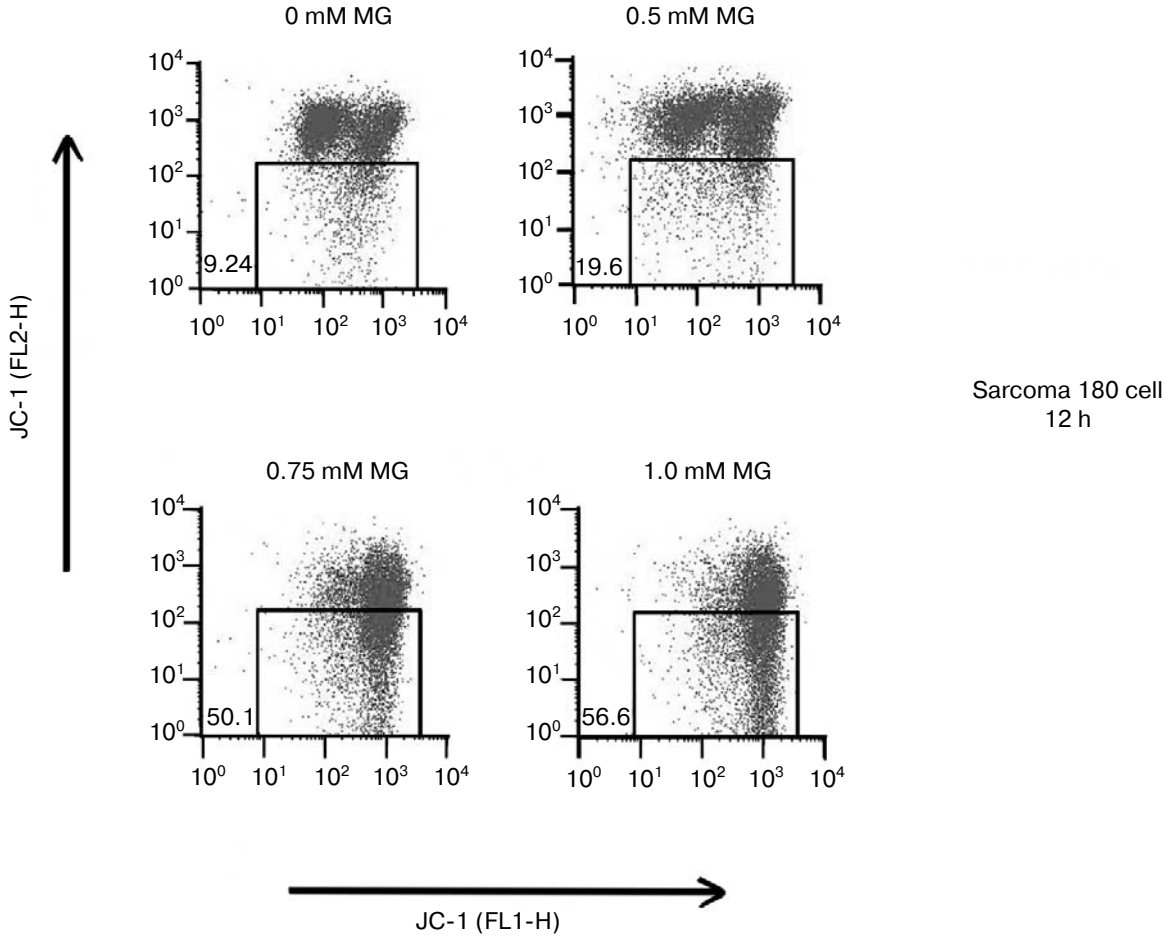

Fig. 6. Mitochondrial membrane potential assays of sarcoma 180 cells by JC-1. Loss of mitochondrial transmembrane potential measured by flow cytometric analysis revealed the shift of red to green fluorescence in dose-dependent manner within 12 h of methylglyoxal treatment.

observed in spectrofluorimetric assay of membrane potential with isolated mitochondria from sarcoma tissue.

The results presented in this and the preceding paper [5] clearly indicate that action of methylglyoxal is selective against malignant cells. This selectivity is observed in the results obtained by different experimental approaches such as polarographic study of mitochondrial respiration, spectrofluorimetric study of mitochondrial membrane potential, cytochrome *c* release, atomic force microscopy of possible structural alteration, and spectrophotometric and polarographic assay of NADH dehydrogenase.

Methylglyoxal showed cytotoxicity to several malignant cells through induction of apoptosis [14, 20-22]. In this study also methylglyoxal induces apoptosis in sarcoma tissue mitochondria only whereas normal mouse skeletal muscle mitochondria remained unaffected. Mitochondria play an essential role in death transduction such that the permeability transition pore opening, collapse of membrane potential, and a rapid release of caspase activator, cytochrome *c* [23]. Reactive oxygen species (ROS) might play a major role because blocking of electron transport through complex I generates ROS, producing oxidative stress in cells which is known as a stimulator of apoptosis [24]. It was previously reported that methylglyoxal-induced ROS generation triggered apoptosis in human Hep G2 cells [21].

All these findings provide evidence that ATP deprivation, membrane potential dissipation, and subsequent cytochrome *c* release trigger the events leading to loss of mitochondrial membrane integrity and intrinsic cell death in sarcoma tissue. However, the precise molecular mechanisms and signal transduction pathway of MG-induced apoptosis need further investigation.

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