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Knockdown of apoptosis-inducing factor disrupts function of respiratory complex I

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ABSTRACT: Recent findings suggest that apoptotic protein apoptosis-inducing factor (AIF) may also play an important non-apoptotic function inside mitochondria. AIF was proposed to be an important component of respiratory chain complex I that is the major producer of superoxide radical. The possible role of AIF is still controversial. Superoxide production could be used as a valuable measure of complex I function, because the majority of superoxide is produced there. Therefore, we employed superoxide-specific mitochondrial fluorescence dye for detection of superoxide production. We studied an impact of AIF knockdown on function of mitochondrial complex I by analyzing superoxide production in selected cell lines. Our results show that tumoral telomerase-positive (TP) AIF knockdown cell lines display significant increase in superoxide production in comparison to control cells, while a non-tumoral cell line and tumoral telomerase-negative cell lines with alternative lengthening of telomeres (ALT) show a decrease in superoxide production. According to these results, we can conclude that AIF knockdown disrupts function of complex I and therefore increases the superoxide production in mitochondria. The distinct effect of AIF depletion in various cell lines could result from recently discovered activity of telomerase in mitochondria of TP cancer cells, but this hypothesis needs further investigation.

Introduction

Apoptosis-inducing factor (AIF) is a protein participating in several forms of apoptotic cell death where mitochondrial outer membrane becomes partially permeable to proteins and AIF is released from intermembrane space into cytoplasm and nucleus (Patterson *et al.*, 2000; Green and Kroemer, 2004). Recent findings suggest that AIF may also play an important function inside mitochondria that is connected to reactive oxygen species (ROS) production or scavenging (Vahsen *et al.,* 2004; Miramar *et al.*, 2001; Candé *et al.,* 2004). Increased levels of ROS lead to oxidative stress and endanger cell life, because ROS can oxidize cellular components such as lipids, proteins, and DNA (Fleury *et al.,* 2002). Oxidative stress is involved in many diseases such as atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease, etc. (Bjelland and Seeberg, 2003). However, ROS can be

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also beneficial as they are used by the immune system as a way to attack and kill pathogens and short-term oxidative stress may be important in prevention of aging by induction of mitohormesis (Gems and Partridge, 2008).

AIF is an evolutionary conserved flavoprotein that shares a high degree of sequence homology with bacterial, plant, and fungal oxidoreductases. The gene coding the human AIF maps to chromosome Xq25-q26 and its expression produces a precursor polypeptide of molecular weight ~67 kDa. This precursor contains the N-terminal MLS protein which is cleaved and active AIF (~57 kDa) is created in the mitochondrial intermembrane space (Susin *et al.,* 1999). AIF is probably bound by its N-terminus to surface of the inner mitochondrial membrane (Varecha *et al.*, 2007; Uren *et al.*, 2005). The C-terminus is oriented into the intermembrane space (Otera *et al.,* 2005). AIF, as a NADH oxidase, was proposed to accept an electron from NADH and transfers it to molecular oxygen and thus to create superoxide radical (Miramar *et al.,* 2001). The mechanism of electron transfer by AIF is similar to bacterial ferredoxin reductases, however the structure of AIF is different (Maté *et al.,* 2002). AIF is probably an important component of respiratory chain complex I (Vahsen *et al.*, 2004) which is major producer of superoxide radical (Brand *et al.,* 2004).

Complex I drives the transfer of electrons from NADH to ubiquinone and protons from matrix into the intermembrane space. The complex is formed from more than 40 proteins (Smeitink *et al.,* 2001). Under normal conditions, low levels of produced reactive oxygen species (ROS) are reduced by antioxidant enzymes. Molecule of peroxide is produced from superoxide by magnesium-dependent superoxide dismutase (MnSOD) located in the mitochondrial matrix (Weisiger and Fridovich, 1973). The depletion of functional AIF could result in impaired activity of the complex I (Vahsen *et al.,* 2004). The study carried out using Harlequin mutant mice with significantly decreased AIF expression showed that neurons were more susceptible to apoptosis induced by peroxide in comparison to cells from normal, non-mutant mice (Klein *et al.,* 2002). Therefore, decreased AIF expression led to increased apoptotic death of neurons. Also *in vitro* experiments with cardiomyocytes of Harlequin mice show increased sensitivity of cells to oxidative stress (van Empel *et al.*, 2005). However, possible role of AIF is still controversial because of published conflicting results. For example, knockdown or knockout of AIF in cancer cell lines may result in ROS levels to increase (Apostolova *et al.,* 2006) or decrease (Urbano *et al.,* 2005). It was also previously shown that increased ROS production can be caused by a dysfunction of the respiratory chain and by lower effectiveness of antioxidant mechanisms (Pitkanen and Robinson, 1996).

In this work, we studied the impact of AIF knockdown on mitochondrial complex I function through the analysis of the superoxide production in some selected cell lines that differed in origin (either tumoral or non-tumoral) and in their expression of telomerase. IMR-90 is a non-tumoral (thus telomerase-negative) cell line from lung origin (Akagi *et al.*, 2003). U-2 OS and SAOS-2 cell lines originated from osteosarcomas and are telomerase-negative cells, though they show the alternative mechanism of telomere lengthening (ALT) (Mo *et al.*, 2003; Jegou *et al.*, 2009). A third group of cells are tumoral telomerase-positive (TP) cell lines: the adenocarcinoma cell line HeLa (Harley *et al.*, 1990), the neuroblastoma cell line SK-N-SH (Jain *et al.*, 2007), and the fibrosarcoma cell line HT-1080 (Rasheed *et al.*, 1974). As mentioned above, superoxide level could be used as a valuable measure of complex I function, because most of superoxide production occurs there.

Materials and Methods

Cell culture

The cell lines used were obtained from CellBank, Australia (U-2 OS and SAOS-2) and LGC Standards (HeLa, HT-1080, IMR-90 and SK-N-SH). Cells were grown in minimal essential medium MEM (Pan Biotech) containing EBSS (Earle's balanced salts), L-glutamine, NEAA (non-essential amino acids), 1.5 g/l NaHCO3, 10% fetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37ºC in a 5% CO2 atmosphere. Cells were grown in Lab-Tek II coverglass chambers (Nunc).

Gene silencing by shRNA plasmids

We used shRNA plasmids obtained from SABiosciences. The package contained 4 plasmids specific to various regions of AIF mRNA. Using BlastN search, we chose the plasmid that selectively silenced only AIF mRNA variants and not other proteins. Plasmid was delivered into cells by lipofection using Lipofectamine LTX (Invitrogen). Cells were transiently transfected with shRNA vector for 2 days before analysis. Control cells were prepared by transient transfection with an empty shRNA plasmid for 2 days, as well.

MitoSOX Red staining and fluorescence analysis

Cells were stained with 5 μM MitoSOX™ Red (Invitrogen), mitochondria-specific superoxide detecting red fluorescent dye (emission max. 580 nm), for 10 min at 37ºC as suggested by the manufacturer. After incubation cells were washed three times in PBS (140 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4, 2 mM KH_2PO_4 in distilled water, pH 7.4) and fresh culture medium was added. Flow-cytometry analysis was conducted on trypsinized but living cells using FACScalibur cytometer (BD Biosciences) using channel FL3-H. Confocal 3D image data acquisition was done using Nipkow pinning disk fluorescence confocal microscopy system assembled in our laboratory (Varecha *et al.,* 2007, 2009; Kozubek *et al.,* 2004). Cells were grown, stained, and assayed inside Lab-Tek II coverglass chambers (Nunc).

Image analysis

Image analysis was conducted using free software ImageJ (NIH). For each analyzed cell, we selected one mitochondria-rich z-section of acquired confocal 3D image data where we specified region of interest (ROI) in mitochondrial area of the cell. We measured maximum values of fluorescence intensity inside this region. ROI was defined to contain only regular signals. Maximum value was chosen after several test experiments with various forms of intensity values analyzed (median, mean, etc.). These values were rejected, because they are not comparable among cells and experiments due to variable size of the ROI and particularly due to variable amount of mitochondria in each ROI. Because we chose maximum intensity values for analysis, we had to carefully watch and omit erroneous pixels, noisy signals, and other misleading signals during selection of ROIs. Each analysis was conducted on 50-70 cells and whole experiment was repeated thrice for every cell line. Obtained intensity values were analyzed and graphed in SigmaPlot statistical software (Systat Software). P-values (calculated probability) were estimated using an unpaired Student's t-test. The level of significance was set at $P<0.05$.

Results

To study a role of protein AIF in respiratory complex I, we employed the superoxide-specific fluorescence dye MitoSOX Red to analyze production of the superoxide radical in mitochondria of control and AIF knockdown cells. Cells were transiently transfected with shRNA plasmid to knockdown AIF. AIF shRNA plasmid was selected using BlastN search to selectively silence only AIF mRNA variants and not another protein. Once in the mitochondria, MitoSOX Red reagent is oxidized by superoxide and exhibits red fluorescence. For each experiment we also prepared control cells transfected with an empty shRNA vector in the same way as AIF-silenced cells. Therefore, effectiveness of AIF knockdown was always compared to fresh control cells prepared and assayed under same conditions together with sample cells in the second culture chamber of same LabTek II dish. Thus, if for some reason AIF knockdown transfection would not be successful, we would see no significant difference in obtained results between sample and control cells.

Selected adherent cell lines were chosen to represent a sample of TP cells (HeLa, SK-N-SH, HT-1080), non-tumoral cells (IMR-90), and ALT cells (U-2 OS, SAOS-2). We first conducted flow cytometry analysis of living AIF-knockdown U-2 OS cells using FL3-H channel (Fig. 1). We found that superoxide production was different in AIF knockdown cells in comparison to control cells transfected with an empty shRNA plasmid. Median value was 123 in control cells and 70 in AIF-silenced cells (Fig. 1).

Because MitoSOX Red dye has a tendency to translocate and accumulate in nucleus after being oxidized, we moved from flow cytometry to more selective confocal microscopy of mitochondrial sections only. In this way we were also able to rapidly conduct our experiments, in just minutes after cell staining with MitoSOX

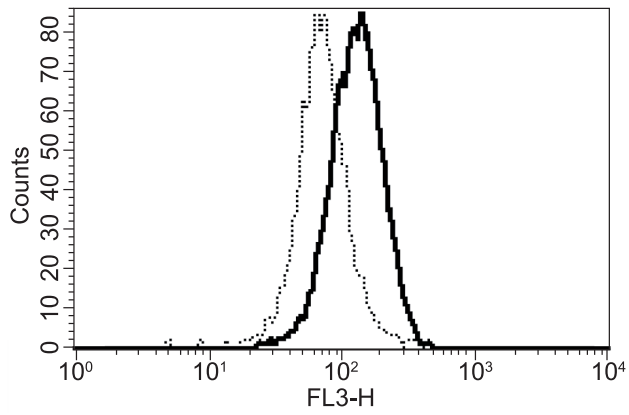

FIGURE 1. Decrease in superoxide production analyzed by flow-cytometry (FL3-H channel) of MitoSOX Red fluorescence intensity in living U-2 OS cells silenced for AIF (dotted line) in comparison to control U-2 OS cells (simple line).

Red. Image analysis of the acquired fluorescence was initiated by finding a region of interest (ROI) in each cell that included a representative mitochondrial area of the cell (Fig. 2). From this ROI we calculated maximum value of fluorescence intensity for each cell.

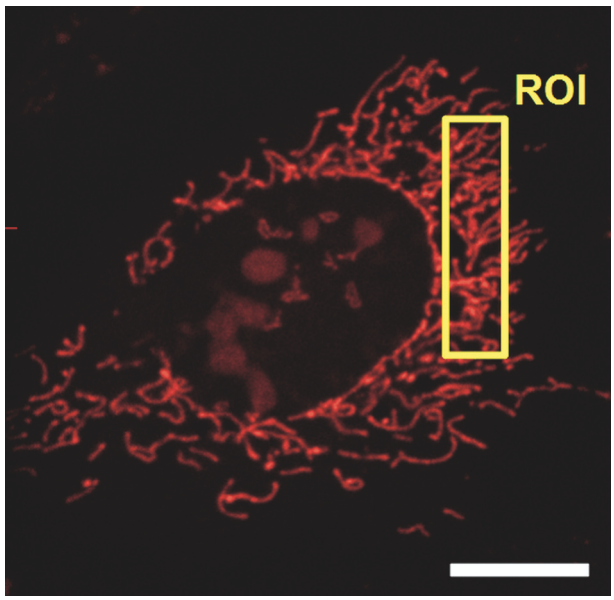

FIGURE 2. Fluorescence microscopy image analysis example. Representative z-section of confocal fluorescence image of living U-2 OS cell stained with MitoSox Red dye. Yellow box represents an example of region of interest used for image analysis. Scale bar represents 10 μm.

The obtained image data were calculated and normalized as percent of control values (Fig. 3). Data clearly show that all three tumoral TP cell lines (HeLa, HT-1080, SK-N-SH) that were tested exhibited an increase in superoxide production after AIF knockdown. On the contrary, the non-tumoral cell line IMR-90 and tumoral ALT cell lines (U-2 OS, SAOS-2) displayed a decrease in the superoxide production after AIF knockdown.

Discussion

In this work, we studied a possible role of protein AIF in mitochondria under non-apoptotic conditions. We conducted many experiments with variety of cell lines to understand the possible involvement of AIF in respiratory chain complex I superoxide production which is tightly connected to condition of the whole complex (Brand *et al.,* 2004). So far, the published results about the role of AIF in mitochondria are contradictory (Miramar *et al.,* 2001; Apostolova *et al.,* 2006; Klein *et al.,* 2002; van Empel *et al.,* 2005; Urbano *et al.*, 2005).

Our results show that increased superoxide production in comparison to control cells was found only in TP cell lines (HeLa, HT-1080, SK-N-SH), while decreased superoxide production was detected in telomerase-negative non-tumoral (IMR-90) and tumoral ALT cell lines (U-2 OS, SAOS-2). All tested cell lines

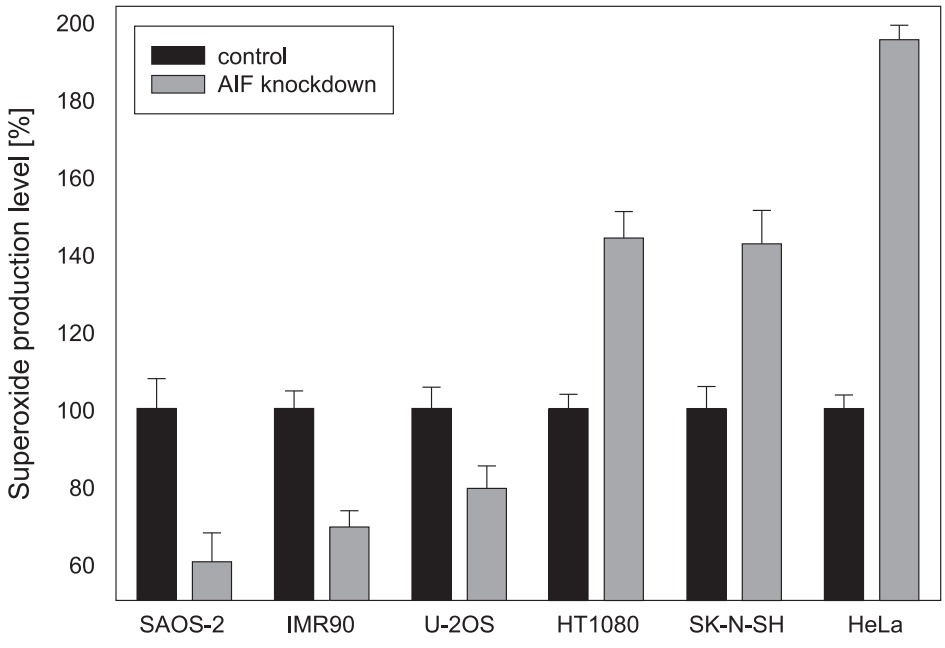

FIGURE 3. Effect of AIF knockdown on superoxide production by several cell lines. Mean maximum intensity values in regions of interest for each analyzed cell were calculated and normalized as percent of control values. Each analysis was conducted on 50-70 cells and the whole experiment was repeated thrice for every cell line.

originate from various tissues. Our results clearly points to an importance of telomerase presence in these cells.

It was observed that telomerase localizes also to mitochondria (Haendeler *et al.,* 2009) and nuclear telomerase translocates into mitochondria upon oxidative stress (Ahmed *et al.,* 2008). Telomerase function inside mitochondria is still a controversial theme, but some authors showed a supportive effect of telomerase on mitochondrial DNA damage during oxidative stress insult (Santos *et al.,* 2004). According to recent results, telomerase binds to mitochondrial DNA coding for complex I genes and increases complex I respiratory efficiency (Haendeler *et al.,* 2009). Also novel results from yeast suggest that enhanced mitochondrial respiration may be mediated by increased number of oxidative phosphorylation complexes per organelle (Shadel and Pan, 2009). It is not clear yet, if this is also valid for mammalian cells and which complexes could be affected.

It has been found that mitochondrial complex I deficiency leads to increased production of superoxide and induction of MnSOD that can significantly reduce the level of superoxide even under control level (Pitkanen and Robinson, 1996). This is probably what we observed for telomerase-negative AIF-knockdown cell lines (IMR-90, U-2 OS, SAOS-2) (Fig. 1, 3). TP cells (HeLa, HT-1080, SK-N-SH) displayed the increased superoxide level after AIF knockdown. We propose that this effect is caused by telomerase involvement in mitochondrial functions. The increase in superoxide production induced by AIF knockdown would lead to oxidative stress resulting in nuclear telomerase translocating into mitochondria. Telomerase could increase copies of respiratory complexes which would lead to even higher production of superoxide. MnSOD function is apparently unable to compensate for the large increase in superoxide level.

We can conclude that AIF knockdown disrupts function of complex I leading to increased superoxide production in mitochondria. We propose that the distinct effect of AIF depletion on observed superoxide production in various cell lines could result from recently discovered activity of telomerase in mitochondria of TP cancer cells. Further studies are needed to elucidate the role of telomerase in mitochondria and thus to test our hypothesis.

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