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Differential action of iodine on mitochondria from human tumoral- and extra-tumoral tissue in inducing the release of apoptogenic proteins

Geeta Upadhyay[a,1], Rajesh Singh[a,2], Ramesh Sharma[b], Anil K. Balapure[b], Madan M. Godbole[a,*]

[a]*Department of Endocrinology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Raebareli Road, Lucknow 226014, India*
[b]*Tissue Culture Laboratory, NLAC, Central Drug Research Institute, Lucknow, India*

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Abstract

Iodide is actively concentrated in the thyroid gland for thyroid hormone biosynthesis. Excess iodine has been observed to induce apoptosis in thyrocytes and mammary cells. The mechanism of iodine induced apoptosis is poorly understood. Among various cell organelles, mitochondria is known to provide conducive environment for the organification of iodine, i.e. iodination of different proteins. Mitochondria also play a central role in execution of apoptosis. To study the role of mitochondria in iodine induced apoptosis, we investigated the direct interaction of iodine and human breast mitochondria vis-a-vis its role in the initiation of apoptosis in vitro. We observed that mitochondria isolated from the tumor (TT) and extra-tumoral tissue (ET) of human breast display significant uptake of iodine. Mitochondrial proteins were observed to be predominantly iodinated in ET but not in TT mitochondria. Treatment with iodine showed an increase in mitochondrial permeability transition of TT and decrease in ET. Iodine induced released factor(s) other than cytochrome c from tumor mitochondria initiate(s) apoptosis in vitro, while those from ET mitochondria were non-apoptogenic in nature. To our knowledge, this is first report demonstrating that iodine acts differentially on mitochondria of tumor and extratumoral origin to release apoptogenic proteins from TT and has a protective effect on ET.
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1. Introduction

Iodide is known to be actively concentrated in the thyroid gland for thyroid hormone (TH) biosynthesis (Carrasco, 1993 and references therein). The ability of the extrathyroidal tissues like salivary gland, gastric mucosa and lactating mammary gland to concentrate iodide has been described since early sixties (Brown-Grant, 1961; Mountford et al., 1986; Spitzweg et al., 2000). Sodium iodide symporter (NIS), the key molecule responsible for the active uptake of iodide in thyroid has been well characterized and its presence has also been demonstrated in extrathyroidal tissues

* Corresponding author. Tel.: +91-522-668-716 ext. 2368; fax: +91-522-668-017/973.
E-mail address: godbole@sgpgi.ac.in (M.M. Godbole).
[1] Present Address: Department of Cellular and Structural Biology, UTHSC, San Antonio, TX 78229, USA.
[2] Present Address: Cell Biology Section, NIEHS, NIH, Research Triangle Park, NC 27709, USA.

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(Dai et al., 1996; Spitzweg et al., 1998, 2000). Thyroid gland is rich in thyroperoxidase, which oxidizes iodide (I^-) to iodine (I_2) during the biosynthesis of TH. Extrathyroidal tissues probably use other tissue specific peroxidases like lacto-, myelo- and eosinophil peroxidase to efficiently organify the iodide (Turk et al., 1983). In the lactating mammary gland, iodide is bound to tyrosyl residues of casein and other milk proteins (Strum, 1978; Shah et al., 1986). These evidences indicate that iodoprotein formation may occur in the non-lactating mammary tissue as well (Strum et al., 1983). The significance of iodination of proteins other than thyroglobulin and casein has not been understood.

Organified iodide regulates the cell cycle in the thyroid gland by inhibiting the transition from G0/G1 and G2/M phase (Tramontano et al., 1989; Smerdely et al., 1993). Excess iodine is also known to induce apoptosis independent of expression of pro- and anti-apoptotic genes in thyrocytes (Vitale et al., 2000). It is known that iodine deficient state renders the rat breast susceptible to dysplasia and carcinoma (Eskin et al., 1967; Eskin, 1970, 1977), phenomena reversible upon inorganic iodine (I_2) administration (Eskin, 1977). Similar effect was also reported in human fibrocystic breast disease (Ghent et al., 1993). It also suppresses 2,7-dimethylbenzanthracene (DMBA) induced breast tumors in the rats both in vivo and in vitro (Ando et al., 1995; Funahashi et al., 1996) probably by the induction of apoptosis (Funahashi et al., 1999). The mechanism of iodine induced apoptosis either in the mammary cells or thyrocytes has not been investigated in detail.

An imbalance between cell proliferation and apoptosis may contribute to tumorigenesis and tumor progression (Merlino, 1994; McDonnel, 1993). Mitochondria are the central executioner of apoptosis and therefore may play an important role in carcinogenesis (Oltavi et al., 1993; Reed, 1994; Green and Reed, 1998). Organification of iodine to proteins requires oxidative enzymes, H_2O_2 generating system and proteins in the vicinity (DeGroot, 1965; Nunez and Pommier, 1982), conditions favorable for the iodination of proteins, which exist in mitochondria under normal circumstances. The direct action of iodine on the mitochondria is still a matter of conjecture owing to lack of evidence(s). This prompted us to investigate the interaction of iodine and mitochondria in regulating apoptosis in tumor tissue (TT) and extra-tumoral tissue (ET).

We investigated the uptake of iodine by the mitochondria and its role in inducing apoptosis. These studies were correlated with the concomitant changes in mitochondrial permeability transition, release of proteins and induction of apoptosis in cell-free system. Cytochrome c which is the initial executioner of apoptosis was also evaluated in iodine induced released factors from TT and ET.

2. Materials and methods

2.1. Subjects

The study subjects consisted of female patients (age 33–58 years) having confirmed fine needle aspiration cytology (FNAC) infiltrating duct carcinoma of the breast (representing tumor node metastasis (TNM) stages I–III). The TT was obtained from the core of the tumor whereas the ET tissue was from mastectomy specimen at least 5 cm away from the tumor margin. Informed consent was obtained from all the patients for the present study. This study was approved by in-house ethical committee.

2.2. Serum analysis

Sera from all the patients were analyzed for thyroid stimulating hormone (TSH), prolactin (PRL) total T_4 and T_3 by standard RIA kits (Diagnostic Product Corp., Los Angeles, CA). All the breast cancer patients were euthyroid and PRL levels were within the normal range for respective age group (data not shown).

2.3. Isolation of mitochondria from human breast tissue

All the steps in the entire methodology reported in the present study were performed at 4°C unless stated otherwise. Human breast tissue from TT and ET was homogenized using a Dounce homogenizer in chilled buffer 'A' (0.32 M sucrose, 1 mM K-EDTA, 10 mM Tris–HCl pH-7.4). The homogenate was spun at $1300 \times g$ for 10 min to remove the cell debris and supernatant containing the mitochondrial fraction was collected. The pellet was resuspended in buffer

'A' to acquire the mitochondria trapped in cell debris for improving the yield. The supernatant obtained from both the steps were pooled and centrifuged at $17{,}000 \times g$ for 15 min to harvest the mitochondria (Harper and Brand, 1993). The mitochondrial pellet was suspended in buffer 'A' and further purified on 1.0/1.5 M sucrose density gradient. The purified mitochondria was collected from the interface and the integrity was assessed by determining the activities of marker enzymes viz. adenylate kinase (ADK, E.C. 2.7.4.3) and citrate synthase (E.C. 4.1.3.7) (Bobba et al., 1999) located in inter-membrane space and matrix respectively. Purified mitochondria were resuspended in appropriate buffer system as per the experimental requirement, was stored on ice and used within 3 h of isolation.

2.4. ^{125}I-iodine uptake in isolated mitochondria

Purified mitochondrial pellet was resuspended at a concentration of 5 mg/ml protein in buffer 'B' (20 mM HEPES-KOH, pH 7.5 containing 68 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, and 1 mM PMSF). An aliquot of mitochondria equivalent to 250 μg protein was treated with 0.1 mCi of ^{125}I for 1 h at 30°C. Mitochondria was separated from the incubation mixture by centrifugation at $15{,}000 \times g$ at 4°C for 10 min and finally washed twice with buffer 'A'. Radioactivity was determined in a gamma scintillation spectrometer (Stratec, USA). Protein was quantified by standard method (Lowry et al., 1951) and iodide uptake was expressed in cpm/mg protein (mean ± SEM).

2.5. ^{125}I-iodine organification assay in mitochondria

The resulting supernatant and pellet obtained after ^{125}I-iodine treatment as described above, by centrifugation at $15{,}000 \times g$ at 4°C for 10 min, were analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were prepared by heating at 70°C for 3 min in $1 \times$ SDS gel loading buffer to denature proteins (10% glycerol, 2% SDS, 62.5 mM Tris–HCl [pH 6.8] with 2-mercaptoethanol). The samples were subjected to electrophoresis at constant voltage of 70 V. The gel was removed, fixed, dried and exposed to X-ray film (Sambrook et al., 1989).

2.6. Iodine induced apoptosis in cell-free system

2.6.1. Isolation of nuclei

Nuclei were isolated from the liver of adult male Sprague Dawley rat using density gradient centrifugation. The liver was homogenized in buffer 'C' (50 mM Tris–HCl, pH 7.4 containing 250-mM sucrose, 25 mM KCl, 5 mM $MgCl_2$) and filtered through four layered cheesecloth. Sucrose (2.3 M) was added to the filtrate to a final density of 1.69 M and under laid with equal volume of 2.3 M sucrose. After centrifugation at $124{,}000 \times g$ at 4°C for 30 min, the nuclear pellet was washed twice with buffer 'C'. It was resuspended in buffer 'D' (10 mM PIPES, pH-7.4 containing 250 mM sucrose, 80 mM KCl 20 mM NaCl, 5 mM sodium EGTA and 1 mM DTT) at a concentration of 8.5×10^7 nuclei/ml and stored at −80°C in multiple aliquots (Blobel and Potter, 1966).

2.6.2. Preparation of the cytosolic extract

Human HeLa-S3 cells were procured from National Centre for Cell Sciences, Pune, India and propagated. Briefly, the monolayer was trypsinized and the cells were harvested by centrifugation as reported earlier by us (Saxena et al., 1995). The cell pellet containing 5×10^5 cells/ml was washed once with ice cold Ca/Mg free phosphate buffered saline and resuspended in 5 vol. of ice cold buffer 'E' (20 mM HEPES-KOH [pH-7.5] containing 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA and 1 mM DTT) supplemented with protease inhibitors (0.1 mM PMSF, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotonin, and 25 μg/ml of ALLN). The cell suspension was kept on ice for 15 min and disrupted by passing 15 times through a 22-gauge needle. After centrifugation in a microcentrifuge at $10{,}000 \times$ g for 5 min at 4°C, the supernatant was further spun at $100{,}000 \times g$ for 30 min in a tabletop ultracentrifuge. The supernatant thus obtained was designated as cytosolic fraction and used in subsequent steps (Liu et al., 1996).

2.6.3. Assay of mitochondrial permeability transition (PT)

To evaluate the in vitro mitochondrial swelling caused by the induction of PT, purified mitochondrial pellet as obtained previously described, was resuspended in buffer 'F' (0.175 M KCl, 0.025 M Tris–

HCl buffer, pH 7.4) at a concentration of 10 mg protein/ml (Scott and Hunter, 1966). An aliquot of 100 μl/ml of mitochondria in buffer 'E' was treated with different concentrations of iodine at 30°C and the absorbance variation caused by swelling was measured at different time intervals using a spectrophotometer at 520 nm.

2.6.4. In vitro release of proteins by iodine treated mitochondria

Purified mitochondria were resuspended at 5 mg/ml in protein release buffer 'F' (20 mM HEPES-KOH, pH 7.5 containing 220 mM mannitol, 68 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM DTT and 1 mM PMSF) and incubated with different concentrations of iodine at 30°C for 1 h. The reaction mixture was centrifuged at $15{,}000 \times g$ for 10 min at 4°C to pellet the mitochondria and the supernate obtained here was designated as released factor(s) and used for further experiments (Liu et al., 1996).

2.6.5. Nuclear morphology and DNA fragmentation by released factor(s) from iodine treated mitochondria

HeLa-S3 cytosol equivalent to 250 μg protein, 6 μl of liver nuclei (8.5×10^7 nuclei/ml), 1 mM $MgCl_2$, 1 mM dATP in a total volume of 50 μl were incubated in the absence or presence of released factor(s) obtained from the previous step at 37°C for 2 h. Following incubation, 5 μl of the reaction mixture was used for evaluating the nuclear morphology by propidium iodide (PI) staining (10 μg/ml) under fluorescence microscope. The percentage of apoptotic nuclei was calculated in five different fields.

Small molecular weight fragments of DNA were isolated from the rest of the nuclei contained in 45 μl reaction mixture from the previous step. Nuclei were resuspended in 500 μl buffer 'G' (100 mM Tris–HCl, pH 8.5 containing 5 mM EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K) and incubated overnight at 37°C. Subsequently, NaCl was added to a final concentration of 1.5 M and centrifuged at $12{,}000 \times g$ for 15 min at room temperature. DNA in the supernatant was precipitated with an equal volume of chilled absolute ethanol, washed with 70% ethanol and resuspended in 25 μl buffer 'H' (10 mM Tris–HCl, pH-7.5 containing 1 mM Na-EDTA and 200 μg/ml DNase free RNase A). Following incubation at 37°C for 2 h the DNA fragmentation was analyzed on 2% agarose gel (Liu et al., 1996).

2.6.6. In vitro assay of cytochrome c release

Purified mitochondria (20 μg equivalent protein) suspended in protein release buffer 'E' was treated with iodine in a reaction volume of 20 μl at 30°C for 1 h. At the end of the incubation, the reaction mixture was centrifuged at $14{,}000 \times g$ for 10 min at 4°C to pellet the mitochondria. The pellet was resuspended in 20 μl of $1 \times$ SDS sample buffer whereas 5 μl of $4 \times$ SDS sample buffer was added to the resulting supernatant. Both samples were electrophoresed on 12% SDS-PAGE under denaturing conditions. The protein was transferred onto a nitrocellulose membrane and probed with a mouse monoclonal cytochrome c antibody (a kind gift from Dr R. Jemmerson, Minneapolis, MN, USA). Antigen/antibody complexes were detected using an HRP-conjugated goat anti-mouse IgG using enhanced chemiluminescence kit (Amersham Pharmacia Biotech, UK).

3. Results

3.1. Breast mitochondria concentrate iodine

^{125}I uptake increased in a dose-dependent manner until 750 nM concentration of iodine and decreased thereafter at 1000–1250 nM in mitochondria from ET. The maximum uptake was observed at 750 nM which was 9-fold more than basal level in ET mitochondria. On the other hand, the uptake in the TT mitochondria which also increased in a dose dependent manner up to 750 nM by only 1.5-fold, attenuating below the basal level at higher concentrations (1000–1250 nM). The basal ^{125}I uptake was 3-fold higher in the TT mitochondria as compared to ET mitochondria (Fig. 1).

3.2. Mitochondrial proteins from breast tissue are iodinated

The above uptake experiments in TCA precipatable fraction suggest that iodine is actively concentrated and organified mainly in ET mitochondria in comparison to TT mitochondria. The factor(s) released from ^{125}I treated mitochondria in the supernatant (referred

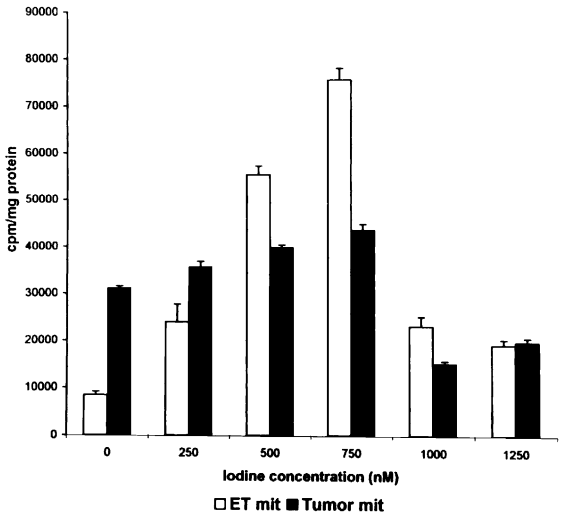

Fig. 1. Iodine (^{125}I) uptake in the mitochondria from tissue in the presence of increasing concentrations of iodine. Mitochondria isolated from TT and ET tissue was purified on sucrose density gradient. Purified mitochondria resuspended in buffer 'B' (250 μg) were incubated with ^{125}I (0.1 mCi) for 1 h at 30°C in the presence of increasing concentrations of iodine. Mitochondria were washed twice with buffer 'A' by centrifugation at 15,000 × *g* at 4°C for 10 min. Radioactivity was measured in mitochondrial proteins precipitated by 10% TCA and results were expressed in cpm/mg protein (mean ± SEM). The experiments were done in triplicate and repeated three times independently.

as 'S') and pellet (referred as 'P') as obtained from iodine organification assay mentioned beforehand, were analyzed on SDS-PAGE to evaluate the iodination of proteins. Iodine was organified predominantly in mitochondrial proteins from ET tissue (Fig. 2A), but not in mitochondria from tumor tissue (Fig. 2B). *In organelle*, iodination of proteins was observed mainly in the fraction 'P' from ET mitochondria of molecular weight 180, 100, 68, 20 and 16 kDa (Fig. 2A). Very light bands of similar iodinated proteins were also observed in TT mitochondria (Fig. 2B). Proteins of 16 and 24 kDa were iodinated strongly as compared to other iodinated proteins in ET mitochondria. However, in the 'S' fraction derived from both TT and ET mitochondria this phenomenon was absent (Fig. 2A: lanes 2, 4, 6 and 8). Different conditions were employed to check for the role of cellular components accomplishing this situation. The presence of additional unlabeled iodine (750 nM) facilitates the iodination of proteins both in TT and ET (Fig. 2A: lane 3, Fig. 2B: lane 3). Incubation with the cytosol abrogates this iodination, except those with low molecular weight viz. 16 and 24 kDa. However, the addition of iodine to the reaction mixture containing cytosol restores the iodination to an extent (Fig. 2A: lanes 5 and 7). We observed the above mentioned phenomenon both in TT and ET however the extent was low in the TT. The ability of HeLa cytosol being iodinated itself was also evaluated. No iodinated proteins were observed in HeLa cytosol (Fig. 2, lane 9).

3.3. Iodination of mitochondrial proteins is peroxidase mediated

To investigate whether iodination of proteins is peroxidase mediated the uptake experiments were carried out in the absence/presence of propylthiouracil

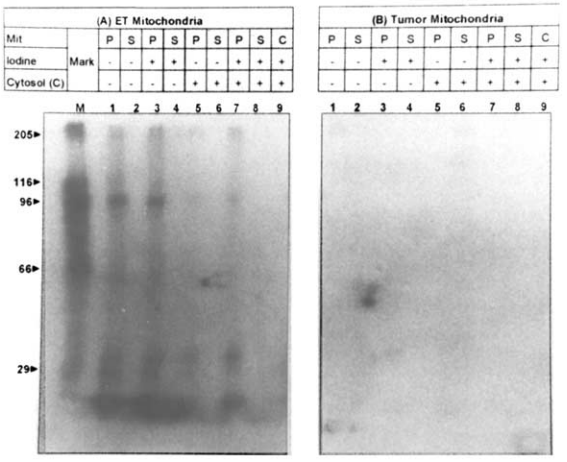

Fig. 2. Analysis of iodinated mitochondrial proteins from ET and TT on SDS-PAGE. Aliquot of isolated mitochondria in buffer 'B' (50 μg) from ET and TT samples were incubated with ^{125}I (0.1 mCi) alone or in the presence of either cold iodine (750 nM) or cytosol or both for 1 h at 30°C. The pellet and supernatant were collected by centrifugation at 15,000 × *g* at 4°C for 10 min. Radiolabeled proteins from both fractions were analyzed on 12% SDS-PAGE. The semidried gel was exposed to X-ray film. A and B depicts the radiolabeled protein analysis pattern from mitochondrial pellet and supernatant of ET (A) and TT (B); without cold iodine and cytosol (lanes 1 and 2), with cold iodine and no cytosol (lanes 3 and 4), with cytosol and no cold iodine (lanes 5 and 6) and with cold iodine and cytosol (lanes 7 and 8). Lane 9 shows absence of radiolabeling of cytosolic proteins. The experiment was repeated three times with tissue samples obtained from three different patients.

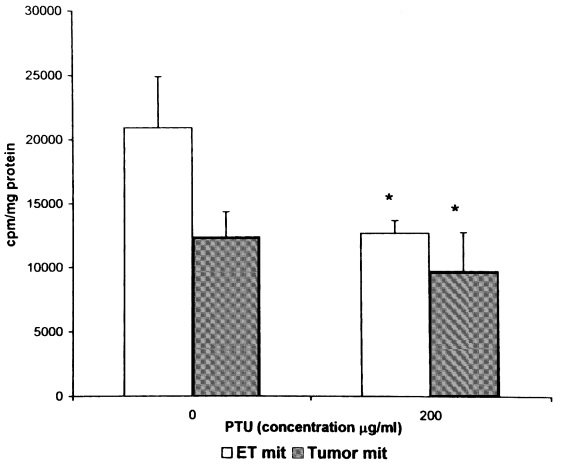

Fig. 3. Iodine (^{125}I) uptake in the mitochondria from tumoral and ET tissue in the presence of PTU. Purified mitochondria resuspended in buffer 'B' (250 μg) were treated with 0.1 mCi ^{125}I for 1 h at 30°C in the presence of PTU. Mitochondria were washed twice with buffer 'A' by centrifugation at 15,000 × *g* at 4°C for 10 min. Radioactivity was measured in mitochondrial proteins precipitated by 10% TCA and results were expressed in cpm/mg protein (mean ± SEM). The experiments were done in triplicate and repeated three times independently.

(PTU), a well known peroxidase inhibitor (Rillema and Rowady, 1997). The dose-response effect of PTU on iodide uptake (data not shown) and incorporation was observed both in TT and ET mitochondria. The basal uptake and incorporation of iodine was more in ET and decreased significantly in the presence of PTU both in TT ($P < 0.05$) and ET ($P < 0.05$) indicating that iodination of proteins is mediated through oxidative enzyme apparatus (Fig. 3).

3.4. Iodine induced permeability transition (PT) in mitochondria from tumor tissue

Extensive iodination of mitochondrial proteins indicated a strong relationship between the two entities. Iodine excess is known to induce apoptosis in thyrocytes and mammary epithelial cells (Vitale et al., 2000; Funahashi et al., 1999). Mitochondria are the central executioner of apoptosis and change in PT is the first step in mitochondrial mediated apoptosis. Therefore, to explore the physiological significance of interaction of iodine and mitochondria, we evaluated mitochondrial swelling as a measure of PT. Permeability transition was measured as decrease in O.D. at 520 nm. The reduction in permeability transition at different concentration of iodine shows a complex pattern. Mitochondria from ET undergo permeability transition without iodine treatment while iodine treatment at all concentrations reduces permeability transition. (Fig. 4B). Permeability transition not seen in mitochondria from TT in the absence of iodine treat-

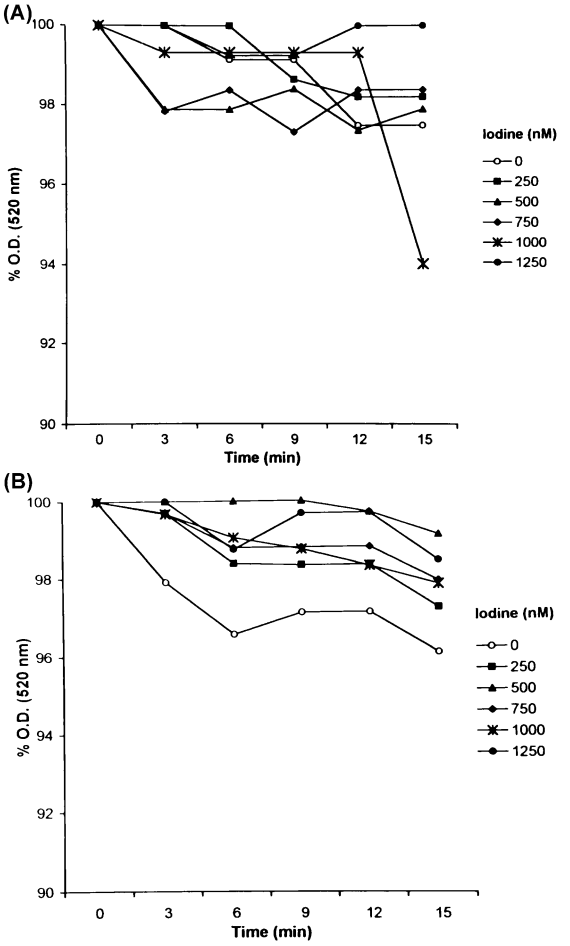

Fig. 4. Optical density (normalized to 100) of a suspension of mitochondria from tumoral and ET tissue as a function of time. Mitochondria from TT (A) and ET tissue (B) were suspended in buffer 'E' at a concentration of 10 mg/ml equivalent protein. An aliquot of 100 μl/ml in buffer 'F' treated with different concentration of iodine (0, 250, 500, 750, 1000 and 1250 nM) and swelling was observed by taking O.D. at 520 nm at different time intervals. The maximum optical density observed was normalized to 100% and percent variations in O.D. were calculated. The experiment was done in duplicate and repeated three times with TT and ET tissue samples obtained from three different patients.

ment and noticeable upon treatment with 500 and 750 nM iodine within 3–8 min (Fig. 4B).

3.5. Iodine induces the release of apoptogenic proteins

To understand whether iodine acts directly on TT and ET mitochondria to induce release of apoptogenic protein(s), an in vitro assay system was developed, originally described by Liu et al. In this system, HeLa cell cytosol was used as a source of procaspases and nuclei from rat liver was used as a substrate for activated caspases. The factor(s) released from iodine treated mitochondria was added to assess its apoptotic property(ies) if any.

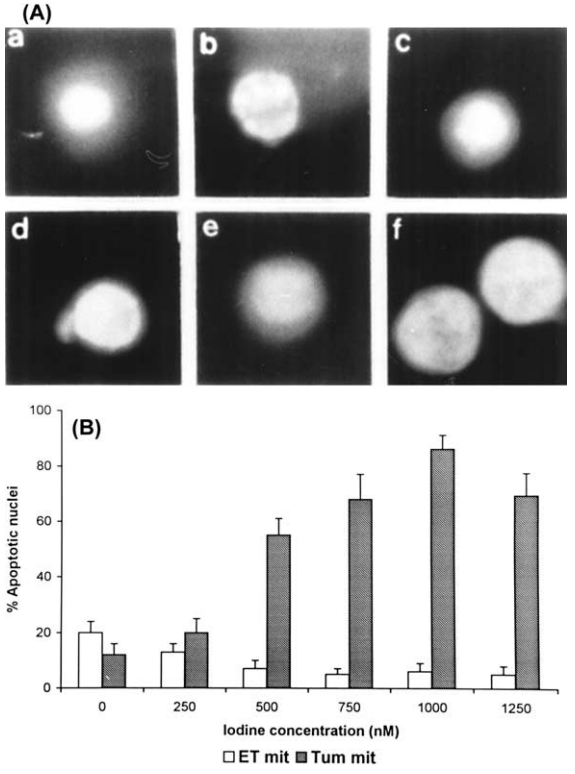

Fig. 5. (A) Nuclear morphology: aliquots of HeLa-S3 cytosol equivalent to 250 μg protein and 6 μl of liver nuclei (8.5×10^7 nuclei/ml) were incubated with iodine (1000 nM) treated mitochondrial supernatant from ET and TT in a reaction volume of 50 μl. The subsection a depicts absence of apoptosis in ET, c–f depict different stages of apoptotic nuclear morphology in TT while b depicts a positive control where liver nuclei were incubated with cytochrome-c in the presence of HeLa-S3 cytosol. Nuclear morphology was observed by PI staining under fluorescence microscope with an UV-2A combination filter. (B) Quantitative analysis of apoptotic nuclei at different concentration of iodine: The apoptotic nuclei were counted in five different views. The experiments were done in triplicate and were repeated three times independently and results were expressed in mean ± SEM.

Upon treatment with the factor(s) released from tumor mitochondria, rat liver nuclei displayed typical features of apoptosis such as nuclear blebbing, protruding bodies, margination and condensation (Fig. 5A) in cell-free system which was not observed from ET mitochondria. The number of nuclei undergoing such changes were dose dependent and being maximal at 1000 nM iodine and decreasing thereafter until 1250 nM. About 70–85% nuclei underwent apoptotic changes at 1000 nM (Fig. 5B). It was interesting to note that released factor(s) from ET mitochondria without any treatment showed apoptotic changes and about 20% nuclei showed this phenomenon and number decreased with increasing dosage of iodine (Fig. 5B).

To further confirm the apoptogenic nature of the released proteins, DNA fragmentation analysis was undertaken. Low molecular weight fragments of DNA were isolated from the nuclei treated with factor(s) released from iodine treated mitochondria from TT and ET. Extensive DNA fragmentation was initiated by released factor(s) in the former (Fig. 6A) whereas only basal fragmentation was observed in the later (Fig. 6B). The extent of fragmentation by released factors from TT mitochondria increased with iodine concentration and was maximal at 1250 nM. It is surprising to observe as in Fig. 6B that even the basal DNA fragmentation observed in lane 2 (released factors from untreated mitochondria from ET + nuclei + cytosol) was further inhibited with increasing dosage of iodine.

3.6. Iodine induced apoptosis is independent of cytochrome c

The release of cytochrome c from mitochondria in response to apoptotic stimuli has been implicated as a major route for the initiation of caspase cascade culminating in cell death (Green and Reed, 1998). To assess whether this sequel of events is true in our case, cytochrome c release assay was performed. The mitochondria from ET tissue demonstrated basal release of cytochrome c. Interestingly however, this basal release was thwarted with the increasing

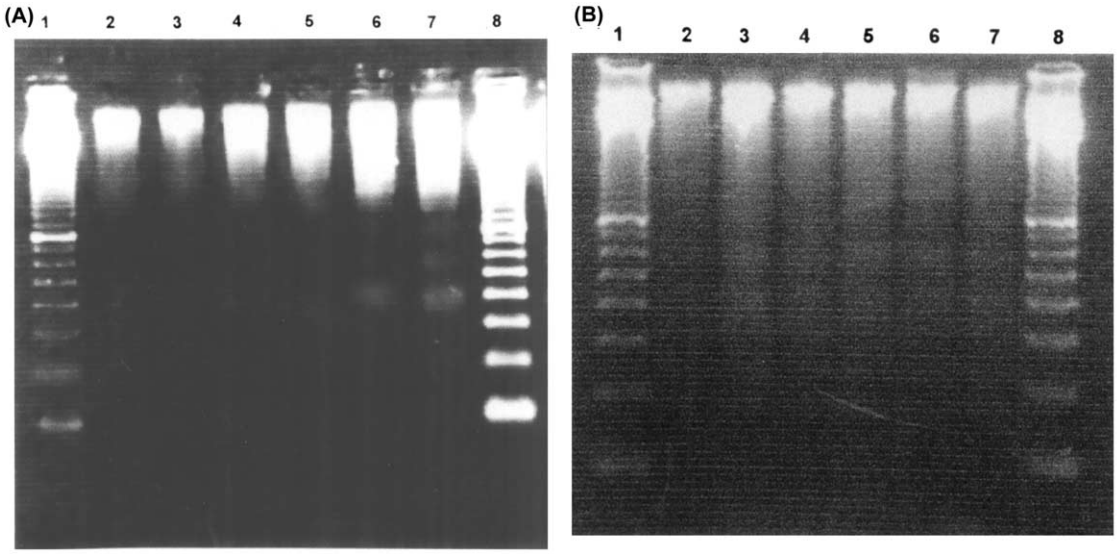

Fig. 6. DNA fragmentation from released factor from iodine treated mitochondrial supernate. Aliquots of HeLa-S3 cytosol equivalent to 250 μg protein and 6 μl of rat liver nuclei (8.5×10^7 nuclei/ml) were incubated with different concentration of iodine with iodine untreated (lane 2) and treated (lanes 3–7) mitochondrial supernate from TT (A) and ET tissue (B) in a reaction volume of 50 μl. Lanes 1 and 8 depicts 200 bp marker while lanes 3–7 show the effects of iodine treatment at concentrations of 250, 500, 750, 1000 and 1250 nM, respectively. DNA was isolated as described in experimental procedure and analyzed on 2% agarose gel.

concentration of iodine and was undetectable finally at 1250 nM in ET mitochondria (Fig. 7B). Conversely, a clear band of cytochrome c was observed and its intensity increased in the pellet with increasing dosage of iodine (Fig. 7B). Cytochrome c release from tumor mitochondria was totally absent both at the basal level or at any concentration of iodine tested and resided exclusively in the pellet (Fig. 7A).

4. Discussion

In the present study we have demonstrated that iodine acts differentially on the mitochondria isolated from human TT and ET breast tissue. The evidence provided here for the first time shows that iodine though taken up by the mitochondria from both TT and ET is organified mainly in the ET. In the tumor mitochondria, protein iodination appears to be minimal and free iodine induces the release of apoptogenic proteins. Iodine seems to play a protective role in ET mitochondria by inhibiting the basal release of cytochrome c and other apoptogenic proteins. The extensive apoptosis induced by proteins released in vitro from TT mitochondria appears to be independent of cytochrome c and implicates the hitherto unknown apoptogenic molecules that need to be studied in greater detail.

For achieving the iodination of proteins, H_2O_2, iodine and oxidative enzymes are *sine qua non* (Ekholm and Bjorkman, 1997). The reactive oxygen species (ROS) including H_2O_2 generated as a by-product of the electron transport chain and oxidative enzymes embedded in the inner mitochondrial membrane make the mitochondria ideal cellular organelles for the iodination of proteins (Ekholm and Bjorkman, 1997; Spitzweg et al., 2000). No evidence of either in vivo or in vitro iodination of protein(s) in mitochondria has so far been reported in the literature, even in classical and established iodination system of the thyroid gland. Therefore, to understand the interaction of iodine and mitochondria, if any, we conducted experiments with ^{125}I and mitochondria isolated from TT and ET regions of the breast tissue.

The mitochondria isolated from TT and ET actively concentrated iodine and is organified in the protein pellet as the counts were taken in the TCA percipitable fraction. This organification of iodine increased with

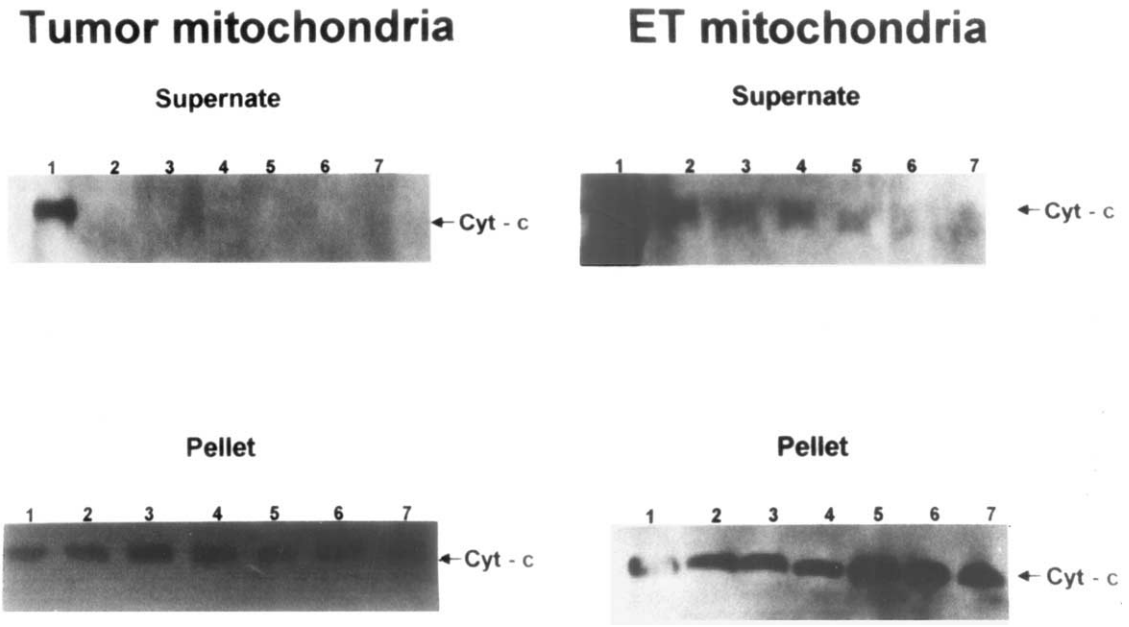

Fig. 7. Analysis of iodine induced release of cytochrome c from mitochondria from TT and ET tissue. Purified mitochondria from TT and ET tissue equivalent to 20 μg protein in buffer 'F' was treated with different concentration of iodine in a reaction volume of 20 μl at 30°C for 1 h. The reaction mixture was centrifuged at 14,000 × *g* for 10 min at 4°C to pellet the mitochondria. The resulting supernatants were mixed with 5 μl of 4 × SDS sample buffer. Both pellet suspension and supernatant were electrophoresed on 15% SDS-PAGE and presence of cytochrome c was analyzed by western blotting. A (both supernatant and pellet from TT) and B (both supernatant and pellet from ET) depict results of western blots. Cytochrome c (lane 1, positive control), and effect of iodine treatment on release of cytochrome c from mitochondria at 0, 250, 500, 750, 1000 and 1250 nM concentrations (lanes 2–8, respectively).

dosage of iodine both in TT and ET, which was significantly higher in ET mitochondria as compared to the TT mitochondria (Fig. 1). This pattern was observed until 750 nM iodine which subsequently decreased both in TT and ET at higher iodine concentration (1000–1250 nM). This phenomenon is similar to Wolf-Chaikoff effect observed in the thyroid tissue where lower concentration favors organification and excess is inhibitory (Wolff and Chaikoff, 1948).

Thus, in vitro ^{125}I uptake experiments suggest that iodine is not only concentrated by the mitochondria but is also organified. The analysis of mitochondrial proteins on SDS-PAGE under denaturing conditions revealed lower iodination in TT mitochondria as compared to ET. The presence of additional unlabeled iodine facilitates the oxidation of I^- by initiating the formation of free radicals and therefore intense iodination in ET was observed (Gentile et al., 1995) indicating parity as in the thyroid tissue. The higher levels of glutathione peroxidase and other anti-oxidative enzymes present in the cytosol (Ekholm and Bjorkman, 1997) possibly prevent the oxidation of iodine by mitochondrial enzyme system either from TT and ET (Portakal et al., 2000; Legault et al., 2000). Hence, the organification of iodine is abrogated in the presence of cytosol and due to which barely detectable bands were observed (Fig. 2). Conversely, the addition of iodine possibly negates the anti-oxidative property of cytosol thereby facilitating the organification as indicated by the intense iodinated bands.

It is well known that higher extent of H_2O_2 is generated in the tumor cells and this has also been recently found to be true for the TT of the human breast cancer. The elevated level of ROS may induce enhanced statement of anti-oxidative enzymes, e.g. superoxide dismutase, glutathione peroxidase and catalase (Ray et al., 2000) in TT (Szatrowski and Nathan, 1991; Portakal et al., 2000; Legault et al., 2000). This may simultaneously inhibit the oxidation of iodine, thereby resulting in diminished organification in TT mito-

chondria (Fig. 2B). This phenomenon was found to be reversible in the presence of iodine to an extent. However, in the presence of the cytosol this phenomenon was undetectable further confirming the antioxidative nature of cytosol.

To assess whether the iodination of proteins is a result of peroxidase mediated reaction, the in vitro iodine uptake experiments were conducted in the presence of propylthiouracil, a peroxidase inhibitor (Rillema and Rowady, 1997). Diminished incorporation of the radioisotope observed in the TCA percipitable fractions both in the mitochondrial proteins from TT and ET (Fig. 3) clearly indicates the involvement of peroxidases or similar enzyme system, which may mediate the process.

The physiological significance of mitochondrial protein iodination seen by us has not been reported in the thyroid gland or in breast. The role of mitochondria in initiating apoptosis has been well established (Green and Reed, 1998). Excess iodine has been shown to induce apoptosis in Fischer rat thyroid cell line (FRTL-5) (Vitale et al. 2000). The present observations of active iodine uptake and finding of iodinated proteins in mitochondria lends supports to direct action of iodine on mitochondria. The question whether direct action of iodine alters mitochondrial physiology to initiate apoptosis was also addressed.

We have employed an in vitro apoptosis system where direct cause and effect relationship can be evaluated without any interference from other stimuli (Constantini et al., 2000). Change in the mitochondrial PT is an important event regulating apoptosis (Green and Reed, 1998). The swelling of mitochondria is a colloid-osmotic process observed during permeability transition in vitro and may cause the release of apoptogenic proteins (Green and Reed, 1998). Exogenous iodine treatment seems to induce the permeability transition in TT mitochondria but not in ET mitochondria (Fig. 4). Analysis of complex PT pattern though beyond the scope of current study needs to be worked out in future. The changes seen in the pattern of PT upon iodine treatment are enough to draw only empirical conclusions.

Mitochondria contain a proteinaceous channel called as permeability transition pore complex (PTPC) which regulates membrane permeability (Marzo et al., 1998).The availability of the iodine to the mitochondria from ET may result in iodination of inter-membrane proteins. This may prevent the release of apoptogenic molecules probably through the reduction in PT as indicated by decreased mitochondrial swelling. However in TT, where the extent of iodination was less, the mitochondria were more susceptible to iodine induced PT. Iodine being a big atom can bring about conformational changes in PTPC complex may induce or inhibit the release of mitochondrial proteins. The characterization of iodinated proteins from both TT and ET mitochondria deserves immediate attention to confirm this hypothesis.

Several apoptogenic molecules are translocated from the inter-membrane space of mitochondria to the cytosol to initiate caspase cascade culminating in apoptosis. The release of cytochrome c from mitochondria is the key event, which initiates the caspase cascade (Green and Reed, 1998). To understand the mechanism of iodine induced apoptosis, in vitro assay of cytochrome c was carried out. Increasing concentrations of iodine in ET mitochondria inhibited the basal release of cytochrome c whereas no release was observed in TT at any of the iodine concentrations tested. It is notable that the basal release of cytochrome c from ET mitochondria contributes towards apoptosis in vitro. The number of apoptotic nuclei and the extent of DNA fragmentation observed at the basal level decreased with the iodine concentration and this is in consonance with the observed inhibition of cytochrome c release (Fig. 7). Thus, the extensive apoptosis induced by factors released from iodine treated tumoral mitochondria suggests that iodine induced apoptosis is independent of cytochrome c. The release of other inter-membrane mitochondrial proteins (heat shock protein-10 (HSP-10), second mitochondrial activator of caspases (SMAC), apoptosis including factor (AIF) and several procaspases in response to iodine needs to be evaluated for their known role in apoptosome formation and the initiation of caspase cascade (Adrain and Martin, 2001). Taken together, it appears that the mitochondria from ET are prone to release apoptogenic proteins while those from TT are resistant to it. Iodine helps restore the mitochondrial-mediated apoptosis in TT whereas in ET, apoptosis is inhibited showing its protective effect. Whether this differential response to iodine is generalized phenomena rendering mitochondria to release apoptogenic proteins in all the tumor types needs to be studied.

Contribution of mitochondria in the process of carcinogenesis have been hypothesized since fifties (Warburg, 1956). The clonal amplification of mitochondria having mutated DNA is known in some carcinoma tissues and recent reports elucidating the role of mitochondria in apoptosis further support the hypothesis (Fliss et al., 2000). The experimental evidences provided here suggest that the mitochondria from TT and ET of breast have disparate responses to the external apoptotic stimuli. Whether mitochondria from TT and ET of other organs behave similarly to apoptotic stimuli other than iodine is not known. The present findings reiterate the recent attempts to target mitochondria as a novel target for cancer chemotherapy (Constantini et al., 2000).

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