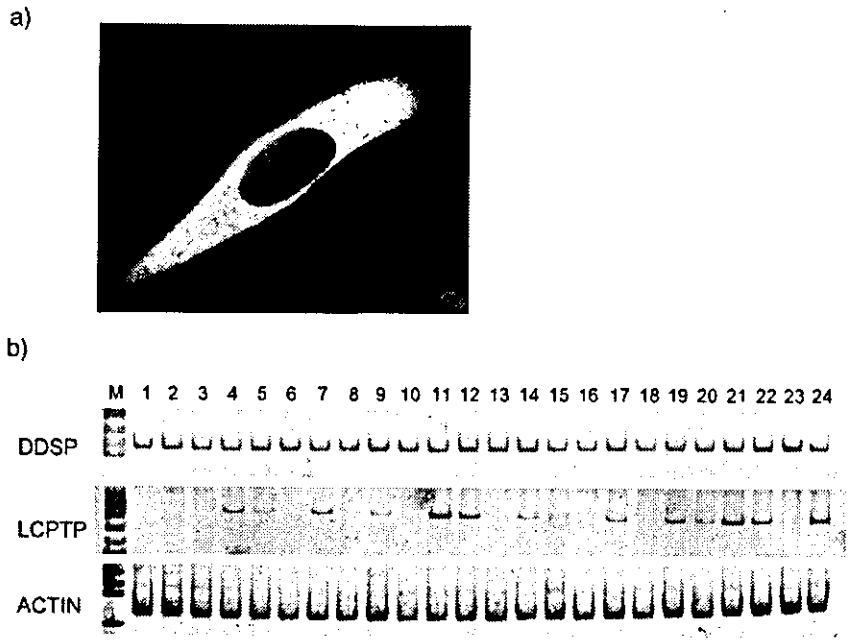

Fig. 3. Subcellular and tissue distribution of DDSP. (a) Cytoplasmic localization of DDSP. The DDSP cDNA sequence was ligated in-frame into pEGFP, transfected into COS7 cells and then subjected to a confocal microscopic analysis. (b) Tissue distribution of DDSP and LCPTP/HePTP. The 2 sequence specific sets of primers for DDSP (top panel) or LCPTP/HePTP (middle panel) were used for RT-PCR. The bottom panel represents the RT-PCR amplifying β-actin mRNA. Lanes are 1: brain; 2: heart; 3: kidney; 4: spleen; 5: liver; 6: colon; 7: lung; 8: small intestine; 9: skeletal muscle; 10: stomach; 11: testis; 12: placenta; 13: salivary gland; 14: thyroid; 15: adrenal; 16: pancreas; 17: ovary; 18: uterus; 19: prostate; 20: skin; 21: lymphocyte; 22: bone marrow; 23: fetal brain; 24: fetal liver.

fected cells with DHEA or treatment of the transfected PEER with or without PMA and A23187 did not significantly change the homogeneous cytoplasmic distribution of GFP-fluorescence (data not shown). Taken together with the observations of the DSP activities, these results indicated that 1–20 encoded a novel member of the cytoplasmic DSP family. We tentatively named this novel DSP DHEA-enhanced DSP (DDSP).

3.4. Profiles of tissue distribution, PMA/A23187 induction and hormonal regulation of DDSP mRNA

To discriminate the mRNA expression of DDSP (full-length 1–20) as potentially DSP from those of tissue-specific LCPTP/HePTP, we designed 2 sets of primers for RT-PCR experiments: one to amplify the sequence specific to DDSP and another specific to LCPTP/HePTP (Fig. 1b). Using these 2 sets of primers, we investigated the tissue distribution and the response to PMA/A23187 or hormonal stimulation. In strong contrast to the restricted tissue distribution of LCPTP/HePTP mRNA preferentially expressed in hematopoietic tissues (and in testis at RT-PCR level), the basal expression of DDSP mRNA was observed by RT-PCR at a similar expression level in all types of human tissues examined (Fig. 3b).

In PEER cells without PMA/A23187 stimulation, the basal expression level (standardized by the fragment length and β-actin expression) of DDSP mRNA was about 20 to 30% of that of LCPTP/HePTP. PMA/A23187 treatment rapidly increased the DDSP mRNA expression within 1 h and then reached a maximum level (5- to 7-fold) at 3 h poststimulation. 100 nM DHEA further increased the DDSP mRNA level by 2.5- to 3-fold at 3 h poststimulation (Fig. 4a). On the other hand, LCPTP/HePTP-specific RT-PCR showed a constitutive expression even in the untreated PEER cells, and PMA/A23187 stimulation with or without DHEA did not significantly alter the expression (Fig. 4b). Costimulation with either 100 nM dexamethasone (DEX), 1 μM DHEA sulfate (DHEAS) or 10 nM dihydrotestosterone (DHT) did not exert the reproducible induction of DDSP mRNA level in the repeated experiments, while the 10 nM 17β-estradiol (E2) treatment sometimes slightly repressed the PMA/A23187-induced DDSP expression. These steroid hormones, including DHEA, did not affect the mRNA levels of LCPTP/HePTP. In Fig. 4c and d, representative results of RT-PCR experiments were shown.

3.5. Interaction of DDSP with the MAPK cascade

To observe the interactions of DDSP with MAPK, we transfected a plasmid expressing a flag-DDSP fusion product into NIH3T3 mouse fibroblasts and then tested the binding of DDSP with activated endogenous ERK1/2, p38-MAPK or JNK by immunocoprecipitation. Western blotting showed that DDSP specifically bound to phosphorylated p38-MAPK activated by hyperosmotic 0.5M NaCl stimulation, but not to

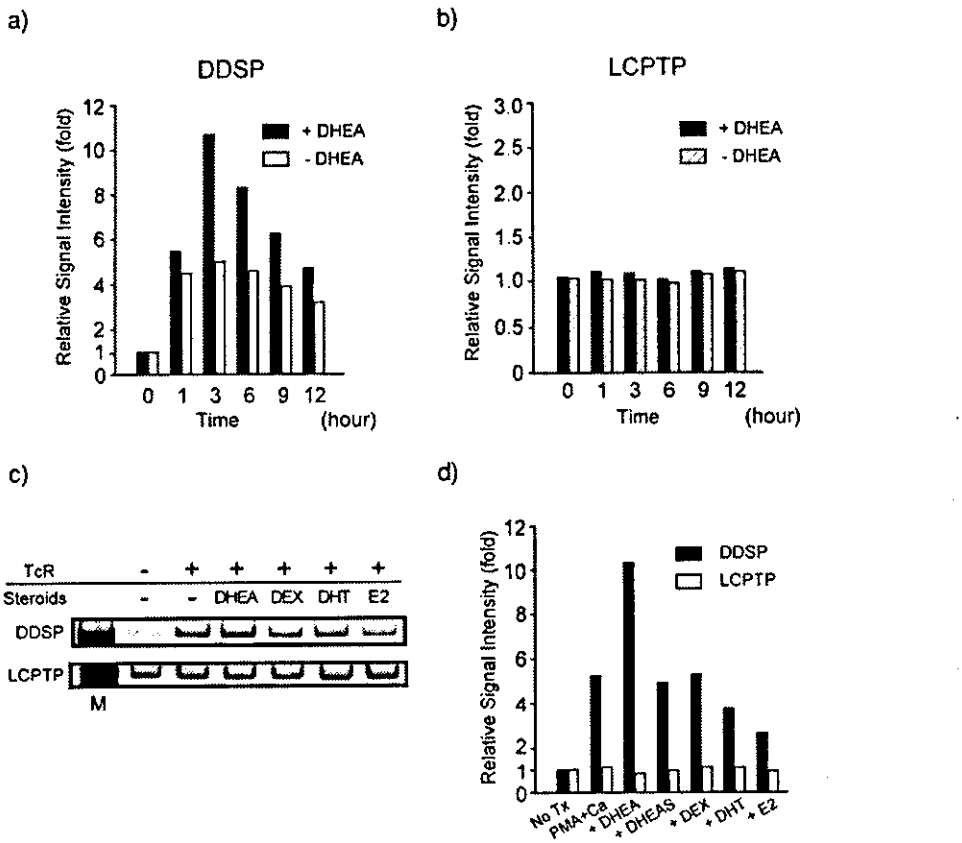

Fig. 4. The differential profiles of mRNA induction between DDSP and LCPTP/HePTP. The same primer set used in Fig. 3b was used for the RT-PCR. (a) The effect of a physiological concentration (100 nM) of DHEA on the expression of DDSP in TcR-activated PEER cells. PEER cells were stimulated by PMA and calcium ionophore A23187 with (filled bar) or without (open bar) DHEA. The relative induction values compared with the basal mRNA expression with no PMA/A23187 treatment, standardized by β-actin expression, are expressed as the-fold induction. (b) Effect of 100 nM DHEA on the expression of LCPTP/HePTP in TcR-activated PEER cells. PEER cells were stimulated as described above. The relative induction values are expressed as in Panel (a). Filled bars: with DHEA; hatched bars: without DHEA. (c) Effects of various steroid hormones on the mRNA expression of DDSP or LCPTP/HePTP. Representative results of the RT-PCR experiments are shown. The PEER cells were treated with PMA and calcium ionophore A23187 in the absence of any steroid hormones, or in the presence of 100 nM DHEA (DHEA), 100 nM dexamethasone (DEX), 10 nM dihydrotestosterone (DHT), or 1 nM 17β-estradiol (E2). The RNAs were extracted after 3 h of treatment and then subjected to semiquantitative RT-PCR using a set of primers specific for DDSP (upper panel) or LCPTP/HePTP (lower panel). (d) Schematic representation of the effects of the various steroid hormones. In this experiment, treatment with 1 μM of DHEA-sulfate (DHEAS) was included. The relative induction of each sequence in the PEER cells compared with the basal mRNA expression with no treatment (No Tx) is expressed as the-fold induction. The PEER cells were treated with PMA and calcium ionophore A23187 in the absence of any steroid hormones (PMA+Ca) or in the presence of 100 nM DHEA (DHEA), 1 μM DHEAS (DHEAS), 100 nM dexamethasone (DEX), 10 nM dihydrotestosterone (DHT) or 1 nM 17β-estradiol (E2), respectively. Filled bars: DDSP mRNA; open bars: LCPTP/HePTP mRNA.

activated ERK1/2 or JNK (Fig. 5a and b). This finding also suggested that the N-terminal 13 aa residues of DDSP (corresponding to aa residues 49–61 of LCPTP) were required for and enough for P38-MAPK-binding.

The inactivation of the phosphorylated p38-MAPK by DDSP was shown by the dephosphorylation of the phosphorylated p38-MAPK. DDSP specifically dephosphorylated the endogenous p38-MAPK, activated by hyperosmotic stimulation using 0.5 M NaCl, in NIH3T3 cells that were transiently transfected with plasmids expressing flag-tagged DDSP, while the phosphorylated ERK or JNK were not dephosphorylated (Fig. 6a). The p38-MAPK-specific inactivation was further confirmed using the pMACS system that can select transfected cells from untransfected cells and can thus enrich the transfected cells to nearly 80% after passage through an affinity column. The expression of the DDSP protein dephosphorylated the endogenous p38-MAPK activated by 0.4 M sorbitol hyperosmotic treatment, but not ERK activated by PMA treatment, in the cells selected after the enrichment (Fig. 6b). Taken together, these results indicated that DDSP inactivates the MAPK cascade in a p38-MAPK-specific fashion.

4. Discussion

To clarify the action of DHEA on a molecular basis, a comparison of the cellular phenotypes between the DHEA-

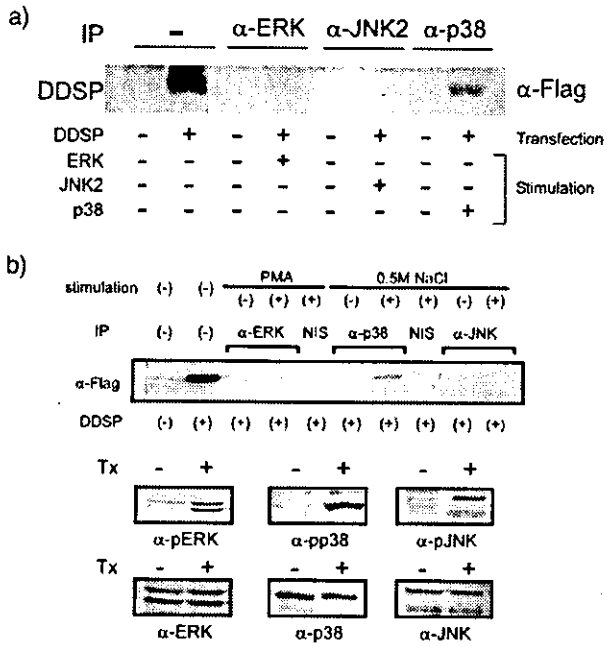

Fig. 5. Immunocoprecipitation of DDSP with ERK, p38 or JNK. (a) NIH3T3 cells transfected with a plasmid expressing flag-tagged DDSP were treated with 0.5 M NaCl for 20 min (for p38 and JNK) or with 50 ng/ml of PMA for 15 min after incubation in a serum free medium for 15 h (for ERK). The endogenous ERK, p-38 or JNK in the whole cell lysates from the treated cells was immunoprecipitated with an anti-ERK, -p38 or -JNK antibody, respectively, and then Western blotting was performed using an anti-flag antibody. DDSP specifically interacts with the endogenous phosphorylated p38-MAPK. (b) Immunoprecipitation and Western blot were performed as in Panel (a) (top panel). NIS: non-immune serum. In the middle and bottom panels, the three pairs of whole cell lysates used for the immunoprecipitations in the top panel were probed to confirm the activation of each MAPK, using antibodies against phosphorylated MAPKs (middle panels: anti-pERK, anti-pp38, anti-pJNK) or total MAPKs (bottom panels: anti-ERK, anti-p38, anti-JNK). Tx: PMA-treatment for the activation of ERK or 0.5 M NaCl treatment for the activation of p38- or JNK-MAPK.

treated and untreated cells led to the isolation of the p38 MAPK phosphatase, DDSP. We demonstrated that this novel member of the PTPN7 locus-derived family was a candidate for one of the target genes of DHEA. One explanation for the biological action of DHEA is that DHEA exerts its functions after being biotransformed into biologically more active androgens and estrogens in either the peripheral tissues or the target cells (intracrine mechanism) [23]. In contrast, the superinduction effect of DHEA on the DDSP mRNA level was specific according to the results shown in Fig. 4. As one of the broad range of actions caused by DHEA, DHEA(-S) has been used for some collagen disease as adjunctive treatment expecting the immune modulating action [24–27] (for review). In this regard, the lymphocytes from the periphery of systemic lupus erythematosus (SLE) patients had a more activated p38 MAPK, as well as ERK or JNK, status immediately ex vivo when compared with lymphocytes from the periphery of normal individuals [28].

We propose a mechanism to explain, at least in part, this immune modulating action, namely that DHEA exerts the anti-inflammatory action by directly suppressing the p38-MAPK cascade. Recently, p38-MAPK has received much attention as a potential drug target for diseases such as rheumatoid arthritis, endotoxic shock, inflammatory bowel disease, osteoporosis and many others [29]. Our findings suggest that DHEA augments the negative feedback regulation of MAPK cascades that have become overactivated due to stress or cytokine signals via a specific set of MAPK phosphatases in many human tissues. In the vascular smooth muscle cell, p38-MAPK activation by PDGF is inhibited by low molecular weight PTPs, thus suggesting that MAPK phosphatases are important negative regulators for the vascular smooth muscle cell growth and migration processes leading to the progression of atherosclerosis [8,9,30]. Apart from the action on the MAPK cascade, it has been demonstrated that DHEA inhibits the nuclear translocation of nuclear factor-κB (NF-κB) probably due to the induction of peroxisome via the peroxisome proliferator activated receptor α (PPARα) [31], or that DHEA inhibits the binding of transcription factor activator protein-1 (AP-1) to DNA [32], thus exerting the anti-inflammatory action. To date, no receptor for DHEA or DHEAS has yet been cloned. The presence of cytoplasmic DHEA binding activity has been demonstrated in human peripheral blood monocytes [7], vascular smooth muscle cell [9] and human T lymphocytes [16], although according to the human genome project, it seems unlikely that a classical steroid hormone receptor-type DHEA receptor exists. Interestingly, Liu et al. reported the existence of the putative membrane receptor for DHEA [33]. DHEA may directly exert its

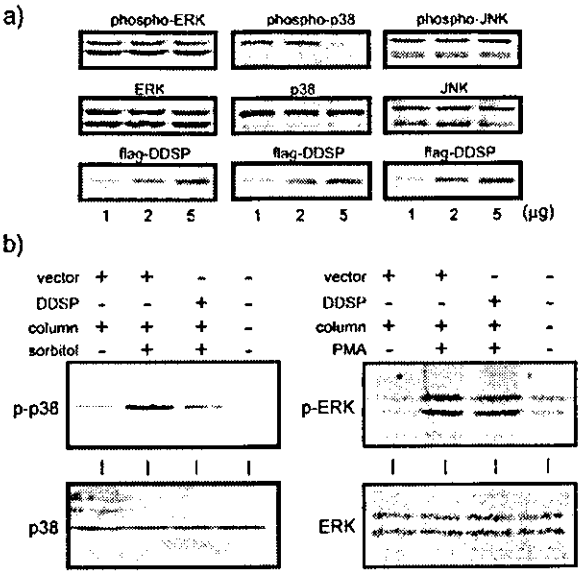

Fig. 6. DDSP dephosphorylates p38-MAPK. (a) Phosphatase activities of DDSP on ERK, p38 or JNK. NIH3T3 cells were transfected with 1, 2 or 5 μg of a plasmid expressing flag-tagged DDSP (left lane, middle lane and right lane, respectively, in each panel). The total amounts of the transfected plasmids were kept constant by adding the empty vector plasmid. The transfected cells were treated as in Fig. 5, and then the whole cell lysates were subjected to Western blotting. The antibodies were raised against phosphorylated (phospho-) MAPKs (top panels), total MAPKs (middle panels) or flag (bottom panels). (b) pMACS experiment showing the p38-MAPK-specific dephosphorylation. NIH3T3 cells were transfected in 10 cm dishes with 20 μg of pMACS-DDSP (DDSP), or pMACSKkII vector (vector) as a control. The transfected cells were enriched after column separation and then treated with 0.4 M sorbitol for 20 min to activate p38-MAPK or with 50 ng/ml PMA to activate ERK. Western blotting was performed using an antibody against phosphorylated p38-MAPK (upper left) or ERK (upper right) (shown as p-p38 and p-ERK, respectively) or total p38-MAPK (lower left) or ERK (lower right) (shown as p38 and ERK, respectively). In this experiment, the column passage alone mildly activated p38-MAPK and caused the basal level phosphorylation of p38-MAPK. The expression of DDSP specifically inactivated the p38-MAPK to the basal level.

action not through the nuclear receptor but through the signal transduction system, such as MAPK system, activated by membrane-type receptors.

The MAPK cascade is regulated by both the phosphorylation and dephosphorylation of the members of the cascade. LCPTP/HePTP has been shown to act as a phosphotyrosine-specific phosphatase for both ERK (ERK2) and p38-MAPK [34,35], and one report showed HePTP as the ERK2-specific phosphatase in the myelogenous leukemia cell line K562 [36]. While LCPTP/HePTP does not dephosphorylate phosphoserine/phosphothreonine residues in ERK1/2 [34], recent findings have revealed that a subfamily of PTP dephosphorylate not only the phosphotyrosine but also the phosphoserine/phosphothreonine residue, and was thus called DSP [37]. When we tested whether or not the DDSP protein possesses phosphatase activities for the phosphoserine/phosphothreonine residue, the DDSP protein also caused a rapid loss of the phosphate from the phosphothreonine as well as from the phosphotyrosine. These results indicated that the DDSP might play a role as a DSP in vivo.

Each DSP or PTP has a restricted subcellular localization [37] (for review), while LCPTP/HePTP has been shown to localize in the cytoplasm (cytoplasmic PTP) as well [38]. DDSP was cytoplasmic as well as LCPTP/HePTP. In contrast, the mRNA profiles of the tissue distribution and the response to MAPK cascade stimulation and steroid hormone treatment were different between DDSP and LCPTP/HePTP, while DDSP was highly homologous to LCPTP/HePTP. Although the mRNA expression of LCPTP/HePTP has previously been shown to be inducible, the RT-PCR experiments using specific sets of primers suggested that the expression of LCPTP/HePTP was constitutive while the actual inducible sequence could be that of DDSP. While mRNA levels in mouse lymphocytes, detected by Northern blots, increased upon stimulation with phytohemagglutinin, lipopolysaccharide, concanavalin A, or anti-CD3 [20], the HePTP protein was present even in resting cells, and its amount increased slightly [34]. The differential mRNA expression between DDSP and LCPTP/HePTP might be due to, though not to be tested yet, the differential promoter usage.

In addition, at the protein level, the substrate specificity was different between DDSP and LCPTP/HePTP. LCPTP/HePTP binds to both ERKs and p38-MAPK through a kinase-interaction motif (KIM) located at the N-terminus of the protein and inactivates them by dephosphorylating the critical phosphotyrosine residue in their activation loop, thus playing a negative role in the TcR signaling pathway [39]. Furthermore, the binding of HePTP to ERK or p38-MAPK is in a phosphorylation-independent fashion [34]. HePTP has previously been shown to interact with both ERK1/2 and p38-MAPK via the 40 aa of the N-terminus sequence [34]. The aa residues 1 to 40 of HePTP corresponded to aa residues 22 to 61 of LCPTP (Fig. 1b). In particular, Arg41 and Arg42 play a crucial role in ERK binding [35]. DDSP lacked the first 48 aa stretch (including Arg41 and Arg42) of LCPTP required for ERK1/2 binding, while the following aa sequence was highly conserved, except for 1 aa residue (Val 220, bold line in Fig. 1b) in addition to the unique 11 aa stretch at the C′-terminus end (Fig. 1a). This may contribute to why DDSP specifically bound to and inactivated phosphorylated p38-MAPK. To date, only one molecule, Wip1, which is induced in response to ionizing radiation in a p53-dependent manner, has been shown to be a p38-specific MAPK phosphatase [40,41]. Our present study suggested the complexity of the gene regulation in the PTPN7 locus. By the mechanisms of alternative splicing and possible differential promoter usage, PTPN7 may encode at least 3 protein phosphatases: one is the inducible DDSP specifically inactivating p38-MAPK and the others are constitutively expressed PTPs inactivating both ERK and p38-MAPK.

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Alterations of Mitochondrial DNA in Common Diseases and Disease States: Aging, Neurodegeneration, Heart Failure, Diabetes and Cancer

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Abstract: It has long been considered that mitochondrial DNA disease is a rare genetic disorder causing neuromyopathy. However, alterations of mitochondrial DNA recently have been recognized to play an important role in the pathogenesis of so-called common diseases such as heart failure, diabetes, and cancer. Although some of these alterations are inherited, more and more attention is being focused on the accumulation of mitochondrial DNA mutations in somatic cells, particularly terminally differentiated cells such as cardiomyocytes and neurons that occurs with age. Mitochondrial DNA is more vulnerable to alteration than nuclear DNA, mainly for two reasons. First, mitochondria are a major source of intracellular reactive oxygen species (ROS). Therefore mitochondrial DNA is under much stronger oxidative stress than is nuclear DNA. Second, mitochondria have a matrix-side negative membrane potential for oxidative phosphorylation. This membrane potential concentrates lipophilic cations inside mitochondria up to ~1,000-fold. Unfortunately, some therapeutic reagents are lipophilic cations, and such exogenously added chemicals are prone to damage mitochondria. AZT, an anti-HIV drug, causes mitochondrial myopathy as a side effect, which is a typical example of how chemotherapeutics adversely affect metabolism of mitochondrial DNA. In this review, we focus on ROS and chemical damage of mitochondrial DNA in common diseases.

Keywords: Mitochondria, mitochondrial DNA, reactive oxygen species (ROS), oxidative stress, aging, DNA damage, DNA repair.

INTRODUCTION

All human cells except for erythrocytes, which are even devoid of nuclei, contain mitochondria. The aerobic ATP synthesis by mitochondrial oxidative phosphorylation is responsible for more than 80% of energy needs [1]. Mitochondria, which possibly evolved from endosymbiotic organisms [2], have their own genome. Mitochondria can semiautonomously replicate and transcribe their DNA. The circular 16.5-kbp DNA of the human mitochondrial genome codes for only 13 subunits of the mitochondrial respiratory chain and a minimal set of 2 rRNAs and 22 tRNAs for constructing the mitochondrial translational machinery (Fig. **1**) [3]. The remaining hundred to thousand proteins in mitochondria are encoded by the nuclear genome, and therefore must be imported into mitochondria [4]. Although they are far fewer in number than the nuclear-encoded proteins, all the proteins encoded by the mitochondrial DNA (mtDNA) are essential for execution of normal oxidative phosphorylation. Thus, disintegration of the mitochondrial genome would cause severe problems with cellular functions and viability. At a cultured cell level, rho^0 cells, which are totally devoid of mitochondrial respiration due to the complete loss of mtDNA, can grow in specially conditioned medium [5]. However, human individuals could not survive without the oxidative phosphorylation of mitochondria. Hence, the maintenance of integrity of the mitochondrial genome with age is critical for the survival of individuals.

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Mitochondria have more functions than just supplying ATP; mitochondria are required for biosynthesis of heme, cholesterol, and phospholipids, all of which are essential cellular components [6]. Furthermore, mitochondria are a key mediator of the initiation of apoptotic events [7] and are the largest source of reactive oxygen species (ROS) [8]. ROS are involved not only in DNA mutations but also in the regulation of intracellular signal transduction pathways leading to cell proliferation [9]. In this regard, the role of mitochondria in carcinogenesis may gain increasing attention.

PRODUCTION OF REACTIVE OXYGEN SPECIES

It has been reported that 1~5% of the oxygen consumed by mitochondria is converted to ROS under physiological conditions [1]. Mitochondrial respiration accounts for about 90% of cellular oxygen consumption, and so the respiratory chain in mitochondria is principally responsible for the physiological production of ROS. Among ROS produced in mitochondria, hydrogen peroxide (H_2O_2) was first reported in pigeon heart mitochondria in state 4 respiration (in the absence of ADP) [10]. Its production is markedly decreased in state 3 respiration (in the presence of ADP), suggesting that the elevated reduction state of components of the respiratory chain enhances H_2O_2 production.

Superoxide anions (O_2^-) are the primary ROS produced in mitochondria (Fig. **2**). Leakage of electrons from the respiratory chain leads to a one-electron reduction of molecular oxygen to O_2^-. H_2O_2 is formed *via* dismutation of O_2^- by superoxide dismutase 2 (SOD2 or MnSOD) present in mitochondrial matrix. The production of O_2^- occurs at two sites in the respiratory chain. One is located upstream of

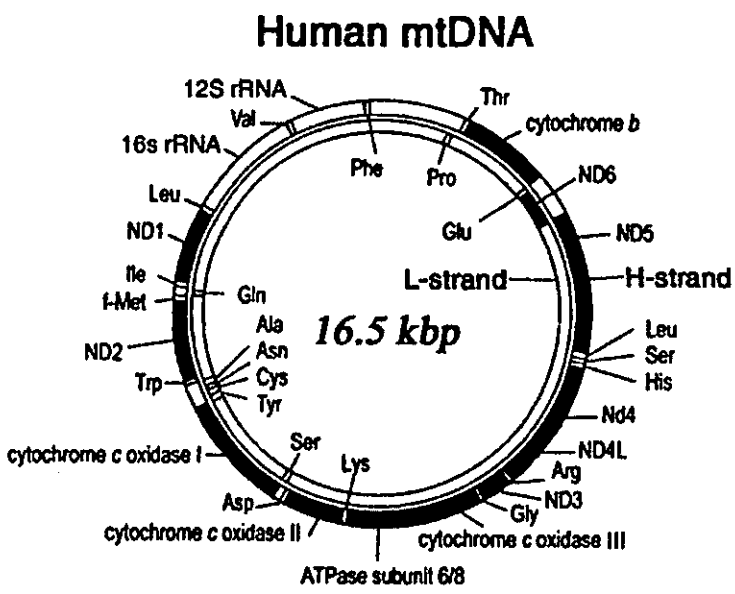

Fig. (1). Structure of human mtDNA.

mtDNA is circular and about 16.5 kbp in length. This gene encodes 13 subunits of mitochondrial respiratory chain, 22 tRNAs, and 2 rRNAs.

the rotenone-binding site of NADH-ubiquinone oxidoreductase (complex I) [11, 12] and the other is between the rotenone-binding site and the antimycin A-sensitive site of ubiquinone-cytochrome *c* oxidoreductase (complex III) [13]. The production of O_2^- at the former site is observed when NADH is used as an electron donor in the presence of rotenone. Rotenone inhibits the electron transport to ubiquinone from complex I by binding to the ubiquinone-binding site or to a nearby site in complex I. This condition makes complex I hyper-reduced, thereby enhancing electron leakage. The formation of O_2^- at the latter site is observed when succinate is used as an electron donor in the presence of antimycin A, an inhibitor of complex III. Under this condition, the production of O_2^- is inhibited by myxothiazol, another inhibitor of complex III. Antimycin A prevents the electron transport to Q^-_o (ubisemiquinone located on the matrix side of inner membrane) from cytochrome b_{562} in the Q cycle, thereby increasing ubisemiquinone. Myxothiazol inhibits the formation of Q^-_o (ubisemiquinone located at the cytosolic side of inner membrane) from reduced ubiquinone (QH_2) by blocking electron transfer to the Rieske iron-sulfur center, leading to an accumulation of QH_2 and decrease of ubisemiquinone. Thus, ubisemiquinone may be responsible for O_2^- formation at complex III [14].

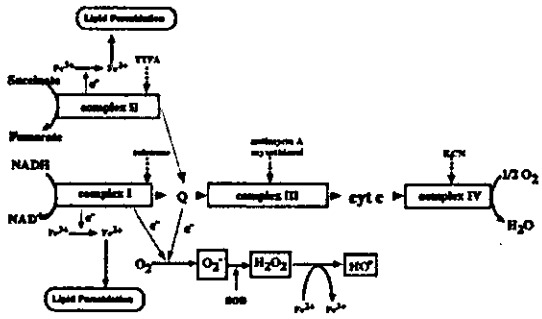

Fig. (2). ROS production in mitochondrial respiratory chain.

Electron leakage for O_2^- production occurs at least two sites: complex I and ubiquinone. The O_2^- could be converted to hydroxyl radicals *via* hydrogen peroxide by Fenton reaction. Electron leakage for reduction of free iron leading to lipid peroxidation reaction occurs in complexes I and II. The leakage sites for lipid peroxidation are different from those for O_2^-.

Lipid peroxide, which is a ROS adduct of lipid, is an acylated form of hydrogen peroxide and also another important ROS produced in mitochondria (Fig. 2). The lipid peroxidation reaction is initiated at two sites of the respiratory chain, complex I [15, 16] and II (succinate-cytochrome *c* oxidoreductase) [17]. Complex I initiates the lipid peroxidation reaction in the presence of rotenone and NADH, while complex II initiates the reaction in the presence of succinate and thenoyltrifluoroacetone, an inhibitor of complex II. Lipid peroxidation is initiated by proton-abstraction by free Fe^{2+} from unsaturated phospholipids. The prerequisite reduction of free Fe^{3+} to Fe^{2+} is performed directly by the respiratory chain, and not by the O_2^-, as evidenced by the fact that (1) free Fe^{3+} is efficiently and directly reduced by the respiratory chain [16], (2) succinate-dependent lipid peroxidation is not accompanied by O_2^- production [17], (3) SOD does not suppress the lipid peroxidation induced by NADH or succinate (unpublished data), and (4) lipid peroxides do not increase in SOD2-knockout mice [18].

The production of both O_2^- and lipid peroxides is initiated by electrons leaked from the respiratory chain. This leakage is enhanced when electron transport is blocked and, as a result, the respiratory chain is placed in a strongly reduced state. This may mimic situations in which mitochondria are impaired *in vivo*. The thiobarbiturate-reactive substances, a marker of lipid peroxidation, increased about two-fold in mouse heart with heart failure, in which complex I activity was decreased [19], suggesting that blockage of electron-flow increases lipid peroxidation *in vivo*. The ROS impair various cellular components including proteins, lipids and DNA. The impaired mitochondrial respiratory chain, in turn, may further enhance the production of ROS. This vicious cycle would be expected to proceed progressively with age [20-23].

MITOCHONDRIAL DNA REPAIR SYSTEM FOR OXIDATIVE DAMAGE

Considering that mitochondria are the greatest source of intracellular ROS and mtDNA is continuously replicated in terminally differentiated non-dividing cells such as nerve cells and myocytes, the repair system for oxidative damage in mitochondria is important. Oxygen radicals, generated through the process of oxidation-reduction reactions in living cells, attack many reactive moieties of DNA. When DNA is subjected to such oxidative stress, strand breaks and base modifications occur, and cellular dysfunction, mutagenesis, and carcinogenesis follow. Among the oxidative lesions of DNA, 8-oxoguanine (8-oxoG), an oxidatively modified guanine base, is the major causative lesion for mutagenesis by oxygen radicals, since it can pair with adenine and cytosine with almost equal efficiency. Thus, 8-oxoG can cause A:T to C:G and G:C to T:A transversion mutations.

Organisms are equipped with elaborate mechanisms to counteract the mutagenic effects of 8-oxoG (Fig. 3) [24, 25]. In *Escherichia coli*, two DNA glycosylases coded by *mutM* and *mutY* genes function to repair 8-oxoG. MutM protein removes 8-oxoG paired with cytosine and MutY protein removes adenine paired with 8-oxoG in DNA. The oxidized form of guanine is also formed in the nucleotide pool of the cell and can be eliminated by the *mutT* gene product. MutT protein hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, thereby preventing misincorporation of 8-oxo-dGMP into DNA [26]. In a mutT-deficient strain, the rate of spontaneous occurrence

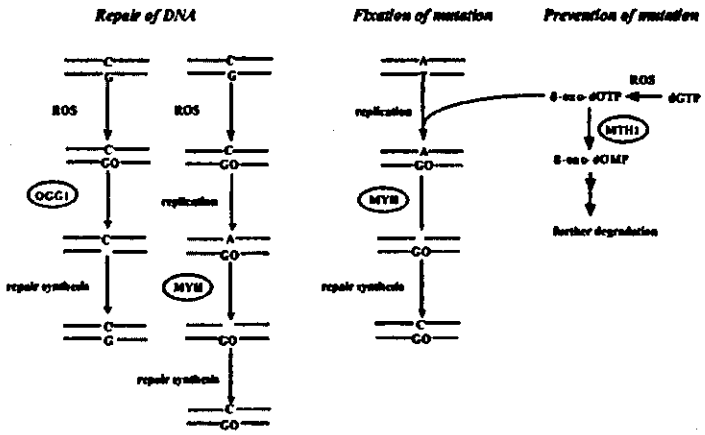

Fig. (3). A repair system for 8-oxoG.

Here, 8-oxoguanine is expressed as GO. Oxidation of a guanine base in DNA produces a C:GO pair. GO of this C:GO pair is removed by OGG1. If the C:GO pair exempts from the repair by OGG1 and enters the replication, the C:GO pair can be converted to A:GO pair. This time, A of the A:GO pair is removed by MYH. Thus OGG1 and MYH co-operate for preventing the GO-caused mutation. On the other hand, 8-oxodGTP, an oxidized from of free dGTP, is also incorporated against adenine base. If MYH removes adenine base of the A:GO pair, the mutation is fixed because the adenine base is a correct base in this case. To avoid this mutation fixation by MYH, 8-oxodGTP must be hydrolyzed by MTH1.

OGG1, 8-oxoguanine DNA glycosylase; MYH, adenine DNA glycosylase; MTH1, 8-oxodGTPase.

of A:T to C:G transversion increases hundreds to thousands-fold compared to the wild type [27]. While the spontaneous mutation rate in a *mutM* or *mutY*-deficient strain is 10- to 50-times higher than that in a wild type strain, the rate of mutation in a *mutM-mutY* double mutants is equivalent to that in the *mutT* mutant [27].

In eukaryotic cells, a pool of dNTP for nuclear DNA replication is present mainly in the cytosol. For mitochondrial DNA synthesis, mitochondria preserve a pool of dNTP, which comprises more than 10% of the total intracellular dNTP [28, 29]. The amount of mitochondrial DNA is roughly 1% of the total DNA in the cell. Judging from their different behaviors, nucleotides in the mitochondrial pool seem to be synthesized in the mitochondria, rather than being derived from the cytosolic pool. Therefore, oxidized dNTPs in mitochondria must be removed in mitochondria. Mammalian cells contain repair enzymes similar to those in *E. coli*. 8-Oxo-dGTPase has been purified from human Jurkat cells, a T-cell leukemia cell line, and its cDNA has been isolated and the genomic sequence determined [30, 31]. The human 8-oxo-dGTPase shows a considerable degree of amino acid sequence homology with *E. coli* MutT protein, and expression of the human cDNA in *mutT*-deficient *E. coli* cells reduces the increased frequency of A:T to C:G transversions to the level seen in the wild type. Hence, the enzyme is considered to be a human counterpart of MutT protein, and the gene has been named *MTH1* (for *mutT* homolog 1).

As described above, the mitochondrial respiratory chain is a major site for the production of O_2^- that can be converted to hydroxyl radicals *via* hydrogen peroxide. The hydroxyl radical is the main species of active oxygen that attacks guanine bases [32]. Thus, DNA and dNTP in the mitochondrial pool may be exposed to greater oxidative stress. The repair of oxidized mitochondrial DNA and elimination of 8-oxo-dGTP from the mitochondrial dNTP pool may be crucial to maintain integrity of mitochondrial DNA. Several lines of evidence show that the mitochondrial matrix possesses 8-oxo-dGTPase, and suggest that the 8-oxo-dGTPase present in mitochondrial and cytosolic fractions is the product of the same gene [33]. The activity of 8-oxo-dGTPase is mainly found in cytosolic and mitochondrial soluble fractions of Jurkat cells. Electron microscopic immunocytochemistry, using a specific antibody against MTH1 protein, shows localization of MTH1 protein in the mitochondrial matrix. Activity in the mitochondria accounts for about 4% of the enzyme total activity, and the specific activity in the mitochondrial soluble fraction is as high as that in the cytosolic fraction. Furthermore, the 8-oxo-dGTPase activities in cytosolic and mitochondrial soluble fractions co-elute with MTH1 protein by anion exchange chromatography, and the molecular mass of the mitochondrial MTH1 protein is equivalent to that of the cytosolic MTH1 protein (about 18 kDa). *In situ* immunostaining of MTH1 protein in HeLa cells expressing transfected MTH1 cDNA demonstrates an increased cytoplasmic signal together with a weak signal in the nucleus, and the overexpressed MTH1 protein is recovered from both cytosolic and mitochondrial fractions. Thus, the 8-oxo-dGTPase encoded by the single *MTH1* gene is localized both in mitochondria and cytosol. Recent studies have revealed that seven different types of mRNAs that differ in their 5' regions are produced from a single *MTH1* gene [34]. Thus, there is the possibility that MTH1 polypeptides with extended N-terminal sequences might be preferentially translocated to mitochondria.

The human homolog of the *mutY* gene (*hMYH*) has been cloned [35]. The *OGG1* gene of *Saccharomyces cerevisiae* encodes a DNA glycosylase that excises 8-oxoG [36]. This OGG1 is a functional counterpart of bacterial MutM, and the human homolog of its gene (h*OGG1*) has also been cloned [36, 37]. Both hMYH [38] and hOGG1 [39] are localized in mitochondria as well as nuclei. All other enzymes required for a base excision repair system are identified in mitochondria [40]. Thus, mitochondria retain a complete set of enzymes for repair of oxidative DNA damage. At present, neither a nucleotide excision repair system nor a mismatch repair system is known from human mitochondria (see [40] for more detailed mitochondrial DNA repair systems).

SOMATIC MUTATION OF mtDNA

mtDNA suffers much more damage than nuclear DNA. Firstly, mtDNA is under much stronger oxidative stress than nuclear DNA, as described above. mtDNA is in proximity to the ROS-generating respiratory chain. The oxidative damage of mtDNA is exemplified by the fact that 8-oxoG accumulates to a greater extent and increases more rapidly in mtDNA than in nuclear DNA though the absolute amount of 8-oxoG in mtDNA had been overestimated [41]. Secondly, mtDNA suffers damage from toxic chemicals much more strongly than does nuclear DNA [42]. To accomplish aerobic ATP production, mitochondria maintain a membrane potential with the matrix side negative. This membrane potential tend to accumulate lipophilic cations inside mitochondria. Mitochondria import lipophilic cations from the cytosol and accumulate these cations up to 1,000-fold [43] although mitochondria have several transport pathways to get rid of toxic foreign molecules. Some medicinal drugs and biologically toxic chemicals are lipophilic and have positive charges. And thus, for example, mtDNA is modified by alkylating agents several 10-fold more than is nuclear DNA [42].

The greater damage naturally would be expected to cause a higher mutation rate in mtDNA. Consistent with this expectation, genes encoded by mtDNA evolve 10-fold faster than nuclear genes [44]. Recently, using denaturant gradient gel electrophoresis (DGGE), very low-level mutations in mtDNA have been detected, and the mutation rate in human mtDNA indeed has been shown to be several hundred-fold higher than nuclear gene mutation rates [45]. Furthermore, Turnbull *et al.* estimated that mutation rate of mtDNA would be about 100-fold higher than that of nuclear DNA by calculating the incidence of cytochrome *c* oxidase activity-negative cryptic cells of colon epithelia [46]. Point mutations in the control/D-loop region of human mtDNA accumulate in an age-dependent manner [47], and age-related large rearrangements of mtDNA have also reported [48, 49]. Somatically occuring variant mtDNA molecules could be amplified depending on conditions and potentially leading to cellular dysfunction with age. DNA polymerase gamma, which is the only DNA polymerase identified in mammalian mitochondria and which is supposed to be responsible for mtDNA replication, has a 3'-5' exonuclease activity for

proofreading [50]. DNA polymerase gamma synthesizes DNA as faithfully as DNA polymerase for nuclear DNA replication, suggesting that the higher mutation rates in mtDNA are due to stronger damage and/or weaker repair activities. Interestingly, premature aging phenotypes are observed in transgenic mice expressing DNA polymerase gamma devoid of the exonuclease activity [51].

KNOCKOUT MOUSE MODELS

Mitochondria are a major source of intracellular ROS production. Therefore, it is expected that clearing the ROS produced in mitochondria is critical for survival. In this regard, Wallace *et al.* created two interesting kinds of mutant mice. One is a *Sod2*-knockout mouse [52]. Mice lacking mitochondrial SOD2 die within the first 10 days of life with dilatated cardiomyopathy. In contrast, mice deficient in cytosolic SOD (SOD1 or CuZnSOD) [53] or extracellular SOD (SOD3) [54] have a very benign phenotype. This suggests that mitochondrially produced ROS is a fundamentally important endogenous oxidative stress. In the *Sod2* mutant mouse, activities of mitochondrial enzymes containing iron-sulfur clusters, aconitase, and succinate dehydrogenase (complex II) are reduced, but not the cytochrome *c* oxidase activity. O_2^- is presumed to react with the iron-sulfur clusters. One of the principal functions of SOD2 appears to be protecting the iron-sulfur cluster-containing enzymes against direct inactivation by O_2^-. By chronic treatment of the knockout mice with SOD mimetic manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP), mean life span was extended from 8 days to 16 days [55]. These results indicate that oxidative stress is a major factor in aging and that synthetic antioxidants can provide therapeutic approaches by alleviating endogenous oxidative stresses.

The other model is a mouse deficient in adenine nucleotide translocase 1 (ANT1) which is expressed in skeletal and cardiac muscles [56]. The *Ant1*-deficient mice developed mild cardiomyopathy. ANT is responsible for importing ADP into the mitochondria and exporting ATP out of the mitochondria. Thus, ANT provides substrate for ATP synthase. Without phosphorylation of ADP at the ATP synthase, electrons do not normally flow in the respiratory chain. Hence, the inactivation of ANT would also cause the block of the electron flow, and the block of the electron flow would enhance the production of O_2^- as already described. SOD2, which is normally induced by oxidative stress, was strikingly increased in the *Ant1* mutant mice. Interestingly, mtDNA rearrangements markedly accumulated in contrast to the *Sod2*-deficient mice. Consistent with this, heterozygous missense mutations have been identified in the human nuclear gene encoding ANT1 in several families with autosomal dominant progressive external ophthalmoplegia (adPEO), which is a rare human disease which presents large-scale mtDNA deletions [57]. O_2^- is converted to H_2O_2 by SOD and then H_2O_2 is converted to hydroxyl radicals *via* Fenton chemistry. Therefore, it is anticipated that hydroxyl radicals increase more in the *Ant1*-mutant mice than in the *Sod2*-deficient mice. Thus, in *Ant1*-deficient mice, hydroxyl radicals would attack mtDNA and enhance fragmentation of it.

HEART FAILURE MODELS

A few % of O_2 consumed in mitochondria is normally converted to ROS, suggesting that the conditions that

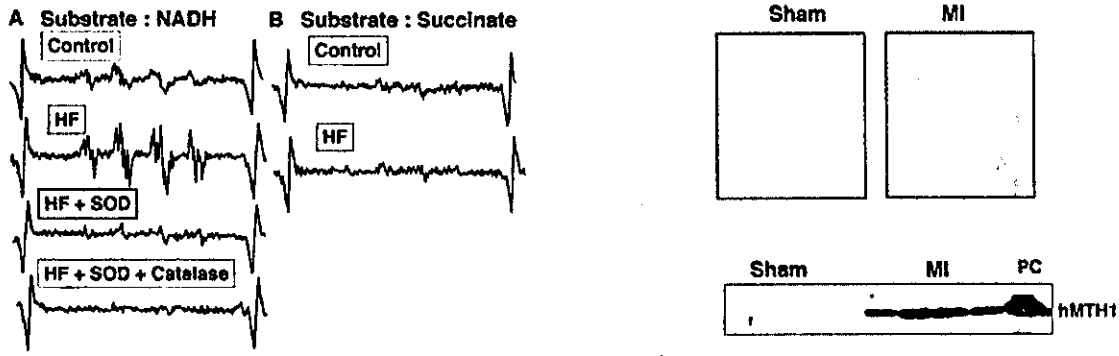

Fig. (4A). O_2^- production in heart failure mitochondria.

Total homogenates of mouse hearts were used for detection of O_2^-. When NADH is used for an electron donor, ESR observed enhanced signals. The ESR signals disappeared with SOD, indicating O_2^- production. Because succinate did not enhanced the O_2^- production, the electron leakage for O_2^- production may occur mainly at complex I. HF, Heart failure.

(B). Upregulated expression of MTH1.

Immunohistochemistry of mouse heart with anti human MTH1 antibodies shows stronger staining in MI heart than in control (upper panel). The MTH1 signals are observed by Western blotting in all five independent MI mouse heart homogenates but not in control heart samples (lower panel).

increase O_2 consumption are oxidative stresses. To verify this, we employed two kinds of cardiac working overload models: hyper-electric pacing and post-infarction hypertrophy.

Dog hearts were forcefully beaten at 240 beats/min using an electric pacemaker. This forceful pacemaking causes heart failure in 4 weeks. The level of lipid peroxides (as a level of thiobarbituric acid-reactive substances) increased in these failing hearts, suggesting that working overload causes oxidative stress [58]. Complex I activity, but not complex II activity, was decreased in the failing hearts. As expected, the mitochondria prepared from the hearts showed an increase in NADH-dependent, but not succinate-dependent, O_2^- production (Fig. 4A). Thus, complex I but not complex II is damaged in this working overload-induced heart failure model.

When partial infarction of a mouse heart is made by occlusion of one coronary artery, the remaining non-ischemic portion must work harder to maintain pumping function. The overload finally leads to heart failure with dilatated cardiomyopathy in 4 weeks. This is another kind of working overload model. The lipid peroxidation level of the hearts was higher in this post-infarction model than in controls, indicating an increase in oxidative stress in the post-infarction heart [19]. Remarkably, the amount of mtDNA in the hearts decreased to about 50% of the control. In parallel with this decrease, the amount of mRNAs encoded by mtDNA decreased. The activities of the complexes I, III, and IV, all of which contain the subunits encoded by mtDNA, decreased. However the activity of complex II, which is not encoded by mtDNA, remained almost at a normal level. These results suggest that a primary target of ROS in post-infarction hearts is mtDNA, not proteins of the respiratory chain [19].

Results in this model of heart failure are reminiscent of the *Ant1*-deficient mice. If O_2^- were responsible for the oxidative damage as in *Sod2*-deficient mice, the iron-sulfur clusters, which are contained in complex II, would be first impaired. Hence, hydroxyl radicals are supposed to be responsible. Electron spin resonance (ESR) was used to detect hydroxyl radicals, in which 4-hydroxy-2,2,6,6-tetramethyl-piperidine (hydroxy-TEMPO) was chosen as a trapper of hydroxyl radicals [59]. The hydroxy-TEMPO *per se* is a radical reagent and therefore shows a characteristic spin resonance signal. It specifically reacts with a hydroxyl radical and then is converted to a non-radical substance or an inert substance on ESR. The post-infarction hearts indeed clearly showed an increased level of hydroxyl radicals [19], and this increase in hydroxyl radicals could explain the primary damage of mtDNA. Interestingly, the expression of MTH1 was upregulated in the post-infarction hearts, presumably serving to prevent oxidative mtDNA damage (Fig. 4B) [60]. In addition, we would like to underscore that the ESR method using hydroxy TEMPO has an advantage in the detection of ROS. In this hydroxy TEMPO-ESR system, hydroxyl radicals, which are supposed to be directly responsible for the oxidative damage, can be specifically detected using total tissue homogenates instead of isolated mitochondria. We propose that mtDNA can be a direct and primary target of ROS under conditions where SOD2 is present or upregulated.

The dilatated cardiomyopathy developed in the post-infarction hearts was improved by long-term treatment with dimethylthiourea (DMTU), a hydroxyl radical scavenger [61]. The fibrosis that is associated with the cardiomyopathy was also reduced with DMTU treatment. These results suggest that oxidative stress is involved in the progression of cardiac dysfunction. It is known that oxidative stress markers are increased in human congestive heart failure patients [62, 63]. Antioxidant therapies could be one approach in the treatment of chronic heart failure patients.

AGE-ASSOCIATED NEURODEGENERATION

Parkinson Disease and Mitochondrial Oxidative Stress

Parkinson disease is one of the most widespread age-associated neurodegenerative diseases with motor

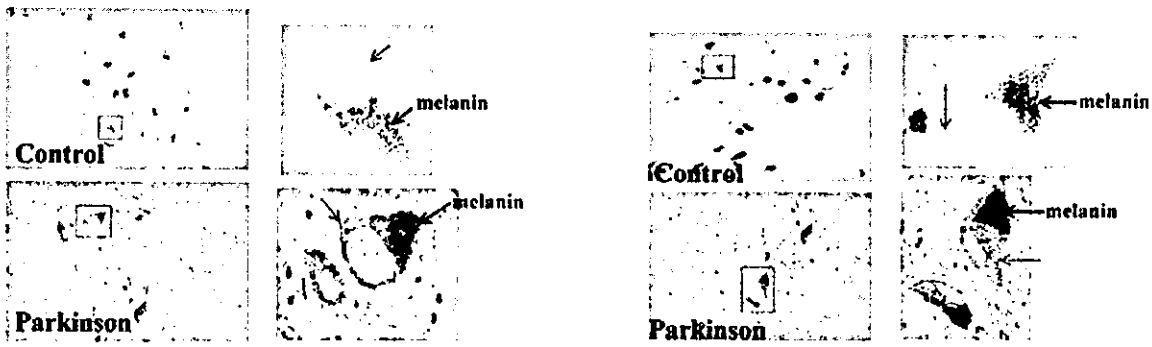

Fig. (5A). Immunostaining of 8-oxoG.

Substantia nigra is immunostained with anti 8-oxoguanine antibodies. Upper panels for control and lower panels for a patient with Parkinson disease. The squared parts are magnified in right panels. The shaded arrow indicate cytosolic staining of 8-oxoG.

(B). Immunostaining of hMTH1.

Substantia nigra was immunostained with anti human MTH1 antibodies. Upper panels for control and lower panels for a patient with Parkinson disease. The squared parts are magnified in right panels. The shaded arrows indicate cytosolic staining of hMTH1.

abnormalities. This disease is caused by a decrease in nerve cells in the substantia nigra. Mitochondrial dysfunction in nigral neurons is supposed to be involved in its etiology and progression of the symptoms [64]. Particularly, a decline of complex I activity is well recognized [64]. The impaired activity of complex I could enhance ROS production. In fact, nigral cells of Parkinson disease patients are intensely stained with anti-4-hydroxynonenal (HNE) antibodies [65]. HNE is a degradation product of lipid peroxides and is frequently used as a marker of lipid peroxidation. Hence, it is suggested that in Parkinson disease the brain is under increased oxidative stress. More mtDNA molecules with a common deletion (4,977-bp deletion with break points at nucleotide positions 8,470 and 13,447) are found in Parkinson disease than in age-matched controls [66], suggesting that mtDNA is also more strongly damaged in this disease. We observed that nigral cells are cytoplasmically stained with anti-8-oxoG antibodies (Fig. **5A**) [67], but that staining is not observed in cortex cells. These results suggest that the oxidative stress and damage is intense in nigral cells in Parkinson disease. The cytoplasmic staining of 8-oxoG also suggests that mtDNA but not nuclear DNA is principally damaged. hMTH1, human 8-oxo-dGTPase, is upregulated in the nigral cells of patients with Parkinson disease (Fig. **5B**). In addition, the upregulation of hMTH1, which is located both in cytosol and mitochondria, is mainly observed in mitochondria [67]. Considering that mtDNA suffers the most oxidative damage, the upregulation of hMTH1 in mitochondria is reasonable in prevention of the 8-oxoG-induced mtDNA mutation. Because the upregulation of hMTH1 or accumulation of 8-oxoG is not observed in multiple systemic atrophy, a disease state with similar depletion of nigral cells, the mitochondrial oxidative stress is not likely to be a result of the depletion of the nigral cells but is instead likely a causative event specific to Parkinson disease. Thus, mitochondrial oxidative stress may play an important role as an etiology in Parkinson disease.

MPP^+-Induced Parkinson Disease Model

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is used to produce an experimental model of Parkinson disease in primates, rats, and mice [68]. Animals that receive MPTP show marked reductions in the number of dopaminergic cells in the substantia nigra pars compacta. MPTP is oxidized in glial cells to 1-methyl-4-phenylpyridinium ion (MPP^+), which is considered to be directly responsible for cell death. MPP^+ is specifically transported into dopaminergic cells *via* dopamine transporters (Fig. **6**). The selective death of dopaminergic cells by MPTP is due to the selective accumulation of MPP^+ in the cells. In addition, MPP^+ is further concentrated in mitochondria due to its lipophilic and cationic nature. MPP^+, like rotenone, is an inhibitor of NADH-ubiquinone oxidoreductase (complex I) in the mitochondrial respiratory chain [69].

The inhibition of complex I causes the production of superoxide radicals (O_2^-) [12] and induces lipid peroxidation [16]. In fact, MPP^+ stimulates the production of O_2^- and initiates lipid peroxidation reaction in isolated bovine heart submitochondrial particles [70, 71]. Hence, ROS are implicated in MPP^+-induced cell death as well as intracellular ATP depletion [72]. Consistent with this

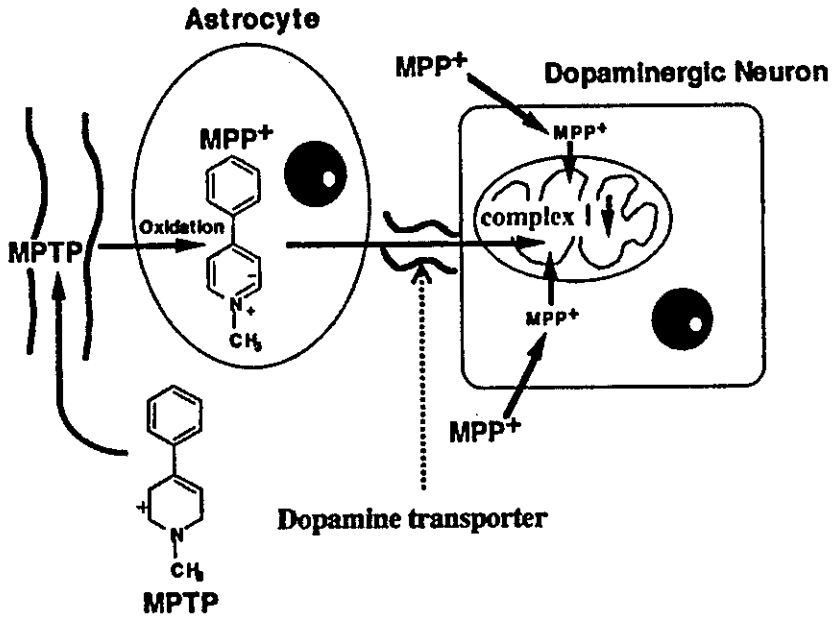

Fig. (6). Scheme for MPP^+ accumulation in a dopaminergic cell.

MPTP in blood is taken by brain astrocytes and then converted to MPP^+ by monoamine oxidase. MPP^+ is specifically transported to dopaminergic cells through dopamine transporters. MPP^+ that accumulates in dopaminergic cells further accumulates in mitochondria according to the inside negative membrane potential, which causes the inhibition of complex I.

notion, transgenic mice overexpressing the copper/zinc type superoxide dismutase [73] and mutant mice lacking neuronal nitric oxide synthase [74] are both resistant to MPTP-induced dopamine neurotoxicity. Thus, MPP^+ is a possible agent of oxidative stress to mitochondria *in vivo*.

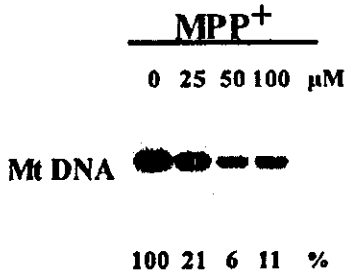

Fig. (7). Decrease in mtDNA by MPP^+ treatment.

Human HeLa cells were cultured with MPP^+ for three days. Then mtDNA was detected by Southern blotting using the same amount of total DNA.

During studies of MPP^+ toxicity, we found that MPP^+ decreases mtDNA in cells (Fig. 7) [75]. The intensity of the 16-kbp band of mtDNA decreased severely in 3 days (Fig. 7), while the intensity of the 10-kbp band of 18S rRNA gene as an internal standard for the nuclear genome was constant, indicating that mtDNA is selectively affected. In these assays, cell growth was fairly maintained and viability was more than 99% by the trypan blue dye exclusion test. Therefore, this decrease in mtDNA is not an event in dying cells. Because we did not observe an increase of smear bands under the 16-kbp band, the decrease of mtDNA seemed not to result from degradation. To determine whether the decrease of mtDNA by MPP^+ is mediated by the inhibition of complex I, we examined the effects of rotenone on the content of mtDNA. MPP^+ is considered to bind to the rotenone-binding site in complex I and thereby to inhibit complex I. In contrast to MPP^+, rotenone increased the content of mtDNA about 2-fold [75]. Thus, the inhibition of complex I alone does not decrease the content of mtDNA.

All evidence so far suggests that mtDNA replication is selectively inhibited by MPP^+. The two strands of mtDNA are called H- and L-strands, respectively. The replication of mtDNA is initiated from the D-loop region, which is a non-coding regulatory region for transcription and replication [76]. The transcription starts downstream from the light strand promoter (LSP) and then the transcript serves as a primer for DNA synthesis of the H-strand (Fig. **8A**). The H-strand synthesis proceeds, displacing the parental H-strand, which forms a characteristic triple-stranded structure, called a D-loop, as a replication intermediate. Frequently, DNA synthesis prematurely terminates and a short nascent strand is formed. This is called a D-loop strand or 7S DNA. To examine the replication states of mtDNA, we specifically amplified the H-strands with free 5' ends in the D-loop region, i.e. nascent strands of mtDNA, by ligation-mediated PCR (LMPCR) (Fig. **8B**, right). To estimate the number of nascent strands per mitochondria, we adjusted the amount of mtDNA between MPP^+-treated and MPP^+-untreated cells for LMPCR analysis. MPP^+ weakened the signals, indicating that the number of nascent H strands per mtDNA decreased (Fig. **8B**, left). These results suggest that MPP^+ indeed inhibits the replication of mtDNA. Thus the inhibition of mtDNA replication is a novel activity of MPP^+ that potentially causes mitochondrial dysfunction independent of the inhibition of complex I.

EtBr [5] and AZT [77] are well-known inhibitors of mtDNA replication. The former inhibits DNA synthesis by DNA polymerase gamma *via* intercalating into DNA strands. The latter is incorporated into DNA as a terminating nucleotide analog. In contrast to these agents, MPP^+ did not inhibit DNA polymerase gamma [78]. Nevertheless, MPP^+ strongly depletes the nascent strands of mtDNA in 6 h after addition to cultured cells [79]. To perform more precise time-course study, we examined the effect of MPP^+ on the amount of nascent H-strands *in organello* using isolated mitochondria [78]. We amplified all H-strands having free 5' ends in the region of O_H, i.e. the total nascent H-strands, by LMPCR. MPP^+-iodide exerted its effect as early as 1 min after addition, and maximally decreased the amount of nascent strands in 5 min. Neither rotenone, an inhibitor of complex I, nor potassium iodide had any effect. The former shows that the decrease is not mediated by the inhibition of complex I. The latter shows that MPP^+ moiety of MPP^+-iodide is responsible for the decrease. Several additional signals were observed at 1 min after the addition of MPP^+ in the LMPCR assay. These additional bands may be degradation products of the D-loop strands released by MPP^+ and suggest that the free D-loop strands are very unstable in mitochondria. As the amount of full-length mtDNA remained constant up to 2 h under these conditions, the decrease of the nascent H strands indicates the release of the nascent strands, i.e., resolution of D-loops which are a putative replication intermediate. Thus MPP^+ inhibits mtDNA replication by resolution of the replication intermediates, D-loops.

In Vitro Effect of MPP^+ on mtDNA Replication

This very fast disappearance of the nascent strands in the presence of MPP^+ strongly suggests that MPP^+ acts on pre-existing D-loops. Hence, we examined whether MPP^+ directly destabilizes D-loop structure *in vitro*. Isolated mtDNA was incubated in the presence of MPP^+, and released nascent strands were detected by Southern blotting. MPP^+ released nascent strands from mtDNA at 10 mM to the same extent as heat denaturation. Ten mM is within a possible range of the intramitochondrial concentration of MPP^+, because MPP^+ has been shown to be concentrated to 20 mM in mitochondria *in vivo* [80]. The third strands in D-loops can be lost by branch migration when negative superhelicity of mtDNA is lost, e.g., by nicking or linearization. MPP^+, however, releases D-loop strands without introducing nicks, suggesting that MPP^+ directly alters the higher structure of D-loops and thereby induces branch migration of D-loop strands. Thus, MPP^+ inhibits mtDNA replication *via* a very unique mechanism, i.e. direct destabilization of D-loops [81, 82].

The most frequently observed forms of DNA damage by ROS are strand breaks, formation of abasic sites, and oxidative modification of bases such as 8-oxoG. It is known

that mammalian mitochondria have repair enzymes at least for the last two forms of damage, e.g. AP endonuclease, uracil DNA glycosylase, and 8-oxoguanine DNA glycosylase (hOGG1) [40]. The last two forms of damage make a single strand break during the repairing process. The D-loop structure depends on superhelicity of mtDNA; relaxation of closed circular mtDNA by nicking releases the nascent strands, i.e. leads to resolution of D-loops. Thus, all three forms of oxidative damage could disrupt a supercoiled structure and release nascent strands. Although MPP^+ can release nascent strands without introducing nicks in mtDNA, ROS produced by MPP^+ [70, 71] could enhance the destabilization of the D-loop. Oxidative stress and impaired mitochondrial functions are implicated in idiopathic Parkinson disease [83]. Thus, destabilization of the D-loop could be involved in the pathogenesis of idiopathic Parkinson disease.

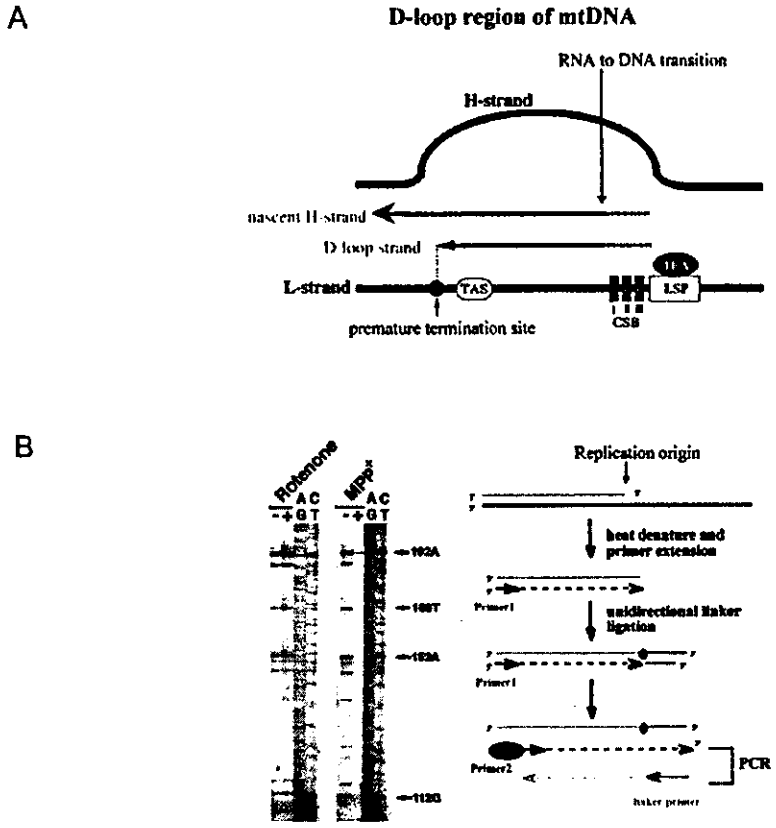

Fig. (8A). Schematic structure of the control region of mtDNA (D-loop region).

TFAM binds to light strand promoter (LSP) and then recruits RNA polymerase, which starts the transcription (shaded broken line). The transcripts are considered to serve as primers for DNA synthesis (shaded straight line). The nascent H-strand synthesis proceeds, displacing the parental H-strand. The DNA synthesis frequently terminates downstream of termination associated sequence (TAS) and forms D-loop strands. According to the model of Clayton, the DNA synthesis that passes through TAS leads to full replication. CSB; conserved sequence block.

(B). Detection of nascent H-strands by LMPCR.

Right panel schematically shows a principle for detection of nascent strands by ligation-mediated PCR (LMPCR). (1) A nascent strand has a free 5' end at a replication origin. (2) Primer extension reaction is done toward the free 5' end, making a blunt end at a site of the original free 5' end. (3) The blunt end is ligated to a double stranded linker that has a blunt end at one side and cohesive end at the other side. (4) After heat denaturation, the nascent strand is amplified using a fluorescent isothiocyanate (FITC)-labeled nested primer 2 and the linker primer. Left panel shows the H-strands that harbor free 5' ends (i.e., nascent strands) by LMPCR. The location and intensity of each signal indicate the location of the free 5' end and the amount of the strand, respectively. Rotenone, an inhibitor of complex I, rather increased the amount of the nascent strands while MPP^+ decreased, suggesting that MPP^+ inhibits the replication independent of inhibition of complex I. The lanes for AG and CT show the sequence ladders created by a chemical cleavage method.

Friedrich's Ataxia

Friedreich's ataxia (FRDA) is a rare neurodegenerative disease accompanied by cardiomyopathy and diabetes [84], the symptoms of which are frequently observed in mitochondrial encephalomyopathy with mtDNA abnormalities [85]. In fact, it is now well-recognized that mitochondrial dysfunction and oxidative stress are causative of this disease. The causative gene for this disease has been identified and designated *frataxin* or *FRDA* [86]. The expansion of a GAA triplet in an intron of the gene is responsible for more than 98% of the patients. It is considered that the triplet expansion induces a triplex and/or quadruplex structure of DNA, and the formation of such non-canonical structures inhibits the transcription of the gene, resulting in a decrease in its gene product frataxin [87]. Targeted disruption of the mouse *FRDA* gene leads to early embryonic lethality. [88], and yeast lacking a frataxin homolog lose mtDNA [89, 90]. Although its exact function is not yet completely understood, frataxin is involved in the homeostasis of mitochondrial iron and/or assembling of iron-sulfur clusters [91]. The resultant iron accumulation and decrease in the activities of mitochondrial respiratory chain complexes enhance mitochondrial oxidative stress and mtDNA damage [84]. Antioxidant therapy is reported to slow the progression of Friedreich's ataxia [92].

mtDNA IN DIABETES MELLITUS

It is well-understood that normal mitochondrial function is essential for insulin secretion in pancreatic beta-cells [93]. For example, when insulin-secreting cells are depleted of mtDNA, they lose the ability to secrete insulin upon glucose stimulation [94]. mtDNA molecules harboring mutations tend to accumulate in non-dividing cells, such as nerve cells and skeletal/cardiac muscles. Diabetes mellitus often accompanies congenital encephalomyopathies with mtDNA defects [85], suggesting that pancreatic beta-cells are also prone to accumulate mtDNA molecules with mutations. An A to G mutation of mtDNA at nucleotide position 3243 (A3243G mutation) is responsible for about 80% of mitochondrial encephalomyelopathy, lactic acidosis, and stroke-like episodes (MELAS), one of the most common forms of mitochondrial encephalomyopathy. Interestingly, 1~2% of diabetic patients, who do not have symptoms of encephalomyopathy, also carry this mutation [95, 96]. Because hundreds to thousands of copies of mtDNA molecules are present in one cell, wild type and mutant mtDNA molecules normally co-exist. This is called heteroplasmy. The degree of heteroplasmy varies among cells and tissues. Defects in mitochondrial respiration do not become apparent until the mutant mtDNA molecules occupy a large part of the total mtDNA population. Those diabetic patients with the A3243G mutation are likely to be a subtype of MELAS patients, where the mutation accumulates mainly in the pancreatic beta-cells. Thus, 1~2% of all diabetic mellitus would be explained by mitochondrial A3243G mutation.

The A3243G mutation is examined most often by using peripheral leucocytes, because it is easy to obtain leucocytes and practically impossible to obtain biopsy samples of pancreas. However, as described shortly, the extent of heteroplasmy is different depending on tissues. The mutant heteroplasmy in leucocytes is known to be much lower than that in muscles in mitochondrial disease. Therefore, there is a possibility that we miss the presence of the mutation due to the low heteroplasmy in leucocytes, and thus underestimate the prevalence of mitochondrial diabetes mellitus. We have developed a more sensitive method, by which 0.01% of heteroplasmy can be detected. However, even applying this method, the A3243G mutation was detected in only ~2% of randomly selected patients with diabetes mellitus [97]. Hence, patients with mitochondrial diabetes mellitus with the A3243G mutation may not exceed 2% of all diabetes mellitus patients in Japan. Even so, this is the biggest single etiological entity identified in diabetes mellitus. In addition, over 200 point mutations have been found so far in mitochondrial diseases. Assuming that all these mutations are potentially a cause for diabetes mellitus, some researchers propose that up to 20% of diabetes mellitus patients may be mtDNA-related.

Impaired insulin secretion in diabetes mellitus may be relevant to impaired mitochondrial respiration in pancreatic beta-cells. The impaired mitochondrial respiratory chain would enhance the oxidative stress. Diabetic hyperglycemia *per se* is a major factor involved in progression of pancreatic beta-cell dysfunction and a variety of pathologic changes [98]. All these complications have been recently shown to be consequences of a single common mechanism, hyperglycemia-induced mitochondrial O_2^- overproduction [99]. In addition, beta-cells are vulnerable to damage caused by ROS at least partly due to very low expression of antioxidant enzyme genes [100]. In fact, in animal models of type 2 diabetes, HNE-modified proteins [101] and 8-oxoG [102] increase. Alloxan, which is used in drug-induced diabetes animal models, causes oxidative mtDNA fragmentation in a mouse beta-cell line [103]. Also streptozotocin rat model of diabetes is related to mitochondrial damage of the beta-cell [104]. Mitochondrial oxidative stress and related mtDNA damage undoubtedly play a critical role in the onset and progression of diabetes mellitus.

mtDNA ALTERATIONS IN CANCER

Mitochondrial aberrations such as altered expression and mutations in mtDNA-encoded products have been identified in a variety of human tumors (see [105] for a review). Recently, it has come to be known that mtDNA mutations are very often found in primary tumors but not in surrounding normal tissues [106, 107]. The mutations are homoplasmic in nature, range over almost entire regions of mtDNA, and include point, deletion, and insertion mutations. This homoplasmic nature of mutated mtDNA raises the possibility that some mutations are involved in tumorigenesis itself by affecting the energy metabolisms and/or ROS production. However, many of the identified mutations are silent in their translation products. These mutations may instead simply reflect the clonal expansion of cells that incidentally have certain mtDNA variants. Statistical calculations indicate that the observed mitochondrial mutations in cancer cells can be explained without assuming any advantage of mtDNA replication or cell survival [108]. The mutated mtDNA found in cancer is 100-fold more abundant than mutated nuclear p53 DNA

[106], indicating that the mitochondrial mutations could be feasible as a molecular marker for detection of cancer.

ANTICANCER DRUGS AND mtDNA DAMAGE

Many chemotherapeutic agents act at the DNA level. DNA-binding chemotherapeutics can inhibit processes such as DNA replication and/or transcription, and thus act as anticancer drugs. Generally, these agents bind to the minor grooves of DNA and intercalate between bases, as examples, actinomycin D and doxorubicin are clinically important DNA intercalators. Mitochondrial DNA is an important target of side effects of these anticancer drugs. One reason is that the mitochondrial respiratory chain can reducibly activate redox-cycling agents and in turn the activated agents produce ROS. Doxorubicin is a member of antracyclines, which consist of quinonoid xenobiotics. Unfortunately, this reagent can produce a characteristic form of cardiac injury involving mitochondrial abnormalities during treatment. It is known that doxorubicin is reduced to a semiquinone radical form by complex I of the mitochondrial respiratory chain, producing a large amount of ROS [109], which can cause oxidative mtDNA damage. In addition, many anticancer drugs will target mitochondria as a side effect because mitochondria accumulate lipophilic cations, as already described. Ditercalinium was originally designed to bis-intercalate into DNA with high affinity, and indeed displays strong cytotoxicity on experimental tumor cells. However, it has turned out after a clinical trial that irreversible hepatotoxicity is a dose-limiting side-effect [110]. This reagent specifically accumulates in mitochondria [110] and depletes mtDNA [111]. As shown in Fig. (9), when mouse cells are incubated with ditercalinium, mitochondria but not nuclei are stained. Importantly, granularly stained materials, which may represent mtDNA nucleoids, are observed in mitochondrial tubular backgrounds, suggesting that ditercalinium accumulates in mitochondria and strongly binds to mtDNA. In the end, this drug was abandoned as a clinical anticancer drug, and is instead used to deplete mtDNA for basic biochemical studies [112, 113]. On the other hand, the potential selective mitochondrial toxicity of lipophilic cations could provide a basis for selective destruction of carcinoma cells [114], particularly because mitochondria are now well recognized to play important roles not only in energy production but also in cell proliferation and cell death.

Fig. (9). Ditercalinium staining of a mouse cell.

Mouse cells were treated with ditercalinium. The self-fluorescence of ditercalinium was observed by confocal microscopy. Granularly stained materials are seen on the mitochondrial tubular background. Each granular signal may represent one mtDNA nucleoid.

CONCLUDING REMARKS

Until recently, interests in alterations of mtDNA were confined to relatively rare inborn mitochondrial encephalomyopathies. Now we have realized that mitochondrial oxidative stress and related oxidative mtDNA damage are critically implicated in wide and common pathological states including aging, cancer, infection, and even medicinal therapies *per se*. However, the molecular mechanisms responsible for maintaining mtDNA integrity have only been poorly delineated. To better understand the causes and consequences of mtDNA alterations in diseases, we need further elucidation of the molecular mechanisms of replication, transcription, translation, repair, and degradation of mtDNA.

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Mitochondrial Transcription Factor A in the Maintenance of Mitochondrial DNA

Overview of Its Multiple Roles

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ABSTRACT: **Mitochondria have their own genome, which is essential for proper oxidative phosphorylation needed for a large part of ATP production in a cell. Although mitochondrial DNA-less (rho^0) cells can survive under special conditions, the integrity of the mitochondrial genome is critical for survival of multicellular organisms. Mitochondrial transcription factor A (TFAM), originally cloned as transcription factor, is essential for the maintenance of mtDNA. Recently, it has become known that TFAM plays critical roles in multiple aspects to maintain the integrity of mitochondrial DNA: transcription, replication, nucleoid formation, damage sensing, and DNA repair. The effects of TFAM in these aspects are intimately related to each other and to function as a whole for the purpose of maintenance of mtDNA.**

KEYWORDS: **mitochondria; mitochondrial DNA; mitochondrial transcription factor A; TFAM; reactive oxygen species; ROS; transcription; replication; DNA repair**

INTRODUCTION

Mitochondria, which probably evolved from endosymbiotically incorporated organisms, have their own genome. Mitochondria replicate and transcribe their DNA semiautonomously. The circular, approximately 16 kbp of the human mitochondrial genome encodes 13 proteins, 2 rRNAs, and 22 tRNAs. All 13 proteins are considered to be essential subunits of a mitochondrial respiratory chain. The rRNAs and tRNAs, used for constructing mitochondrial translational machineries, are also essential for synthesis of the proteins encoded by mitochondrial DNA (mtDNA).[1] Given that the majority of ATP production depends on the aerobic oxidative phosphorylation executed by the respiratory chain, maintenance of the mitochondrial genome is naturally crucial for individuals to survive normally. Mitochondrial transcription factor A (TFAM), which was cloned as a transcription factor for mtDNA,[2] is known to play multiple roles in addition to the transcription in the maintenance of mtDNA. In this

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