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Degraded Mitochondrial DNA is a Newly Identified Subtype of the Damage Associated Molecular Pattern (DAMP) Family and Possible Trigger of Neurodegeneration

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Abstract. We previously showed a preferential degradation and down-regulation of mitochondrial DNA and RNA in hamster fibroblasts in response to hydrogen peroxide. Subsequent studies by others demonstrated that mitochondrial DNA can stimulate immune cells as a DAMP (damage associated molecular patterns) family member. However, the actual physical structure of this mitochondrial DNA DAMP and its importance in non-immune cell types are poorly understood. Here we report that transfected oxidant-initiated degraded mitochondrial polynucleotides, which we term "DeMPs", strongly induce the proinflammatory cytokines interleukin 6, monocyte chemotactic protein-1, and tumor necrosis factor α in mouse primary astrocytes. Additionally, proinflammatory IL1β was induced, implicating DeMPs in inflammasome activation. Furthermore, human cerebrospinal fluid (CSF) and plasma were found to contain detectable DeMP signal. Finally, significant degradation of mitochondrial DNA was observed in response to either a bolus or steady state hydrogen peroxide. Combined, these studies demonstrate, all for the first time, that a pathophysiologically relevant form of mitochondrial DNA (degraded) can elicit a proinflammatory cytokine induction; that a brain cell type (astrocytes) elicits a proinflammatory cytokine induction in response to these DeMPs; that this induction includes the inflammasome; that astrocytes are capable of inflammasome activation by DeMPs; that DeMPs are detectable in CSF and plasma; and that hydrogen peroxide can stimulate an early stage cellular degradation of mitochondrial DNA. These results provide new insights and are supportive of our hypothesis that DeMPs are a newly identified trigger of neurodegenerative diseases such as Alzheimer's disease, which are known to be associated with early stage inflammation and oxidation.

Keywords: Astrocytes, damage associated molecular patterns (DAMPs), degraded mitochondrial polynucleotides (DeMPs), hydrogen peroxide, inflammasome, mitochondria, oxidative stress, proinflammatory cytokine

INTRODUCTION

Elevated levels of reactive oxygen species (ROS) can lead to oxidative stress and subsequent oxidation of all major classes of cellular macromolecules; depletion of intracellular antioxidants and energy

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stores; the release of intracellular stores of calcium; and the inhibition of important cellular processes and signal transduction pathways [1–4]. Many diseases and disorders have been associated with chronic exposure to oxidative stress, including neural disorders, emphysema, arthritis, cancer, atherosclerosis, cataracts, and aging [1, 3, 5, 6]. Mitochondria are especially sensitive to oxidative damage. ATP depletion, loss of electron transport capacity, calcium release, alterations in membrane potential, and mitochondrial-specific lipid peroxidation, protein oxidation, and DNA damage are all consequences of cellular exposure to oxidants [6–9]. This sensitivity is compounded by the fact that the mitochondrial electron transport chain represents a major site of ROS generation. A growing number of diseases have also been associated with mitochondrial genomic alterations and aberrant RNA expression [10, 11].

We previously reported that HA-1 hamster ovary cells down-regulate and degrade both mitochondrial RNA and DNA following exposure to hydrogen peroxide [12–14]. These down- regulations were calcium-dependent, and specific to mitochondria, as cytoplasmic RNAs and nuclear DNA were not degraded under the same conditions. Furthermore, we observed that both growth arrest and apoptosis were associated with peroxide-induced mitochondrial polynucleotide degradation [14, 15]. These results suggested an important role for these degraded mitochondrial polynucleotides in signaling. Subsequent studies by others have demonstrated that mitochondrial DNA can indeed signal; specifically, stimulate immune cells as a DAMP (damage-associated molecular patterns) family member.

DAMP molecules are a relatively recently uncovered family group that trigger immune response [16–23]. Unlike related PAMPs (pathogen-associated molecular patterns), they are able to trigger inflammatory immune responses in the absence of infection. A growing number of DAMPs have been identified, most notably high-mobility group box 1 (HMGB1) protein, S100 protein, heat shock protein, ATP, and DNA. DNA has long been known to be a pathogen-associated molecular pattern agonist [24–26]. However, more recently, mitochondrial (but not nuclear) DAMP DNA has also been found to stimulate an immune response, raising the levels of proinflammatory cytokines and immunomodulators released from immune cells [18, 19, 22, 27–31]. The stimulatory activity of mitochondrial DNA has been found to be due to the presence of unmethylated CpG motifs, which are also present in microbial DNAs. DAMPs are released from damaged and dying cells to serve as danger signals or alarmins, and act by engaging a number of different pattern recognition receptors including extracellular and intracellular toll like receptors, and intracellular inflammasomes. These interactions, in turn, lead to unregulated cytokine and chemokine production as part of immune response [16–23, 27–31]. In the case of inflammasomes, DAMPs act as stimulators that initiate inflammasome assembly, leading to the specific elevation and activation of interleukin (IL)-1 and IL-18 [19, 23, 29, 30, 32]. Several different inflammasomes exist, most notably members of the so-called NLR family. AIM2 is yet another inflammasome type, and is often referred to as the DNA inflammasome due to its ability to sense double stranded DNA from bacteria, virus or even host prior to oligomerization.

It has long been known that circulating mitochondrial and nuclear nucleic acids are present in human blood [33–36]. Importantly, elevated levels of these circulating nucleic acids are found in plasma from patients suffering pathologies such as brain disorders, infection, rheumatoid arthritis, transplantation, and cancer [25, 30–33]. Despite this strong correlation with pathologies, as well as cell based studies that have demonstrated cytokine and immunomodulator induction with mitochondrial DNA [18, 19, 22, 27, 28, 30], the exact physical structure of these important signaling molecules has never been reported. Instead, reported studies have relied on PCR for detection and quantization (e.g., [18, 25, 32, 33]), which does not provide insight into the overall physical structure, and which most likely assumes whole intact mitochondrial genome.

Here, we assess the ability of structurally degraded mitochondrial DNA in triggering immune response using isolated mitochondria from a cell based system (an extension of previous studies on HA-1 cells), and also assess the *in vivo* structural state of mitochondria in human bodily fluids (human cerebrospinal fluid (CSF) and plasma). We observe, for the first time, that degraded mitochondrial DNA is strongly immunogenic; that this immunogenicity occurs in astrocytes and includes inflammasomes; and that circulating mitochondrial DNA exists in a degraded or partially degraded state, suggesting the use of this structural form as a new prognostic and diagnostic marker of disease.

MATERIALS AND METHODS

Cell culture and treatment

HA-1 hamster fibroblasts were generously provided by Dr. Douglas Spitz (University of Iowa) and cultured in minimum essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Primary cultures of type 1 astroglia were prepared from cerebral cortices of 2-d postnatal ES129 mice, essentially as described for rat astrocyte cultures [37], and as approved and described by the Albany Medical College animal care and use committee protocol. Briefly, cortices were dissected and the meninges carefully removed. The tissue was minced then dissociated with 0.25% Trypsin (Invitrogen) in 10 mM HEPES-buffered Hanks balanced salt solution containing 1% DNase for 15 min at 37°C. Dissociated cells were collected by passage through a 70 μm mesh cell strainer into MEM (Invitrogen) supplemented with 0.6% glucose and 10% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Glial MEM), followed by centrifugation for 7 min at 120 × g to remove undissociated tissue and enzymes. Cells were resuspended in Glial MEM and plated at a density of 7.6×10^6 cells per T75 flask. The medium was replaced after 1 d, then twice a week until the cells became confluent (7–10 d). The cells were passaged by brief incubation with Trypsin/EDTA (Invitrogen) at 37°C and frozen in 70% MEM, 20% fetal bovine serum and 10% DMSO. Thawed astroctyes were plated in Glial MEM at a density of approximately 50,000 cells per well and the medium was replaced twice a week until the cultures were 80% confluent (1–2 weeks). This protocol yields nearly pure astrocytes as demonstrated by GFAP immunostaining (not shown). Both cell types were incubated in a humidified incubator atmosphere of 95% air and 5% CO_2 at 37°C.

Isolation of purified and oxidant-initiated degraded mitochondrial DNA

HA-1 cells were subdivided into groups and exposed to hydrogen peroxide (Sigma Chemical Company, St. Louis, MO), glucose oxidase (Sigma; Type X-S from *Aspergillus niger*), or solvent only control. All cultures were then returned to the incubator for the period required. At this time, cultures were removed, scraped, centrifuged, and the cell pellets resuspended in isotonic media (0.25 M sucrose; 20 mM Tris, pH 7;4; and 1 mM EDTA). The pellets were then homogenized and spun 5 min at 1200 × g at 4°C to remove nuclei and unbroken cells. The supernatants were collected and respun under the same conditions. The supernatants were again collected and centrifuged 20 min at 16,000 × g to pellet the mitochondria. The supernatants were discarded and the mitochondrial pellets rinsed once to remove any residual cytoplasm. The mitochondria were then resuspended, lysed, RNAse treated, and DNA purified using QiaAmp columns according to the manufacturer (Qiagen, Valencia, CA).

Quantitation of glucose oxidase-generated hydrogen peroxide

For hydrogen peroxide quantitation, glucose oxidase was added to serum-free HA-1 cell cultures at 50% confluency, and cultures returned to the incubator for the appropriate times. At this time, the amount of hydrogen peroxide present in the media was determined using the hydrogen peroxide detection kit from Enzo Life Sciences (Farmingdale, NY) according to their directions. Sample hydrogen peroxide levels were determined by interpolation of standard curves.

Mitochondrial DNA oxidation status

HA-1 cells were exposed to solvent only (control) or 350 μM of hydrogen peroxide for 2 h, cells collected, and mitochondrial DNA extracted. These DNAs were then dot blotted to nylon membrane, UV-irradiated, and hybridized with antibody specific to oxidized guanine (oxoG) obtained from Cayman Chemical (Ann Arbor, Michigan), followed by peroxidase-conjugated secondary antibody and signal development with Western light chemiluminescent substrate (Perkin Elmer, Boston, MA). Signals were captured on film and quantified using the ImageJ program.

Isolation of DNA from human CSF and plasma

CSF was obtained from the Albany Medical Center Chemistry laboratory under the direction of Jane Noblett from six de-identified patient samples that were centrifuged prior to storage to remove cells. These samples were then combined prior to DNA purification. Plasma DNA was obtained from two volunteers, centrifuged, and the supernatant collected. DNAs were then isolated as above using QiaAmp columns from Qiagen. All human fluid collections were carried out according to the Albany Medical Center Institutional Review Board Protocol.

Cell transfection

The primary mouse astrocytes were placed in 24 well plates for 7–10 days, changing the media every 3 days, and then transfected with the above mitochondrial DNA using 0.4 μg per well using Lifectamine 2000 according to the manufacturer (Invitrogen). Poly dAdT (deoxyadenylic-deoxythymidylic; average size 1220 bases) DNA (Invivogen, San Diego, CA) was transfected in parallel as a positive control. An initial study was carried out to determine the ideal concentration of Lipofectamine 2000, and found to be 2.5 μl per 100 μl MEM. Under these conditions, 35% of cells were successfully transfected as assessed by transfection of green fluorescent protein cDNA. For IL1β analyses, cells were first primed for 5 h with 200 ng/ml *E. coli* LPS prior to transfection. At this time, the cultures were aspirated, rinsed, and replaced with the same whole culture media minus antibiotic just before transfection.

Cytokine analysis

Twenty hours after transfection, cell medias were collected and cytokines analyzed by the Center for Immunology and Microbial Disease Immunology Core facility using the Cytometric Bead Array Flex Sets for IL-6, monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNFα), and IL1β according to the manufacturer (BD Biosciences, San Jose, CA). Cytokine levels from this assay were quantified by interpolation from a standard curve for each cytokine and expressed as pg per ml.

Southern blot analysis

Mitochondrial, CSF, and plasma DNAs were electrophoresed through a 1% agarose gel, then transferred to nylon membrane and hybridized to the entire mitochondrial genome (clone pAM1) labeled with digoxigenin according to the manufacturer (Roche, Indianapolis, IN). A final stringency wash of 0.5X SSC and 0.1% SDS at 65°C was used. Final signals were obtained by exposing to film, and quantified using the ImageJ program.

Statistics

Data is expressed as the mean ± SEM. For cytokine comparisons, the effect of the DNA transfection as compared with mock was determined using a one-sample *T*-test. Statistical significance was concluded when $p \leq 0.05$ for any comparison.

RESULTS

Generation of oxidant-initiated degraded mitochondria DNA from HA-1 hamster fibroblasts

We have carried out many of our oxidative stress studies using an adaptive response cell culture model of HA-1 cells and hydrogen peroxide [12–15, 38, 39], a cell system initially established by Spitz et al. [40]. Among other things, this model is useful for investigating the mechanistic role that oxidative stress plays in various pathological states. Our previous studies included demonstrating that DeMPs are selectively produced in these HA-1 hamster fibroblasts at concentrations of hydrogen peroxide where no cytoplasmic mRNA or nuclear genomic DNA degradation was observed [12–15]. For the present studies, we first repeated our previous DeMP DNA analysis using newly modified conditions to see if they repeated. These modifications included the use of QiaAmp nucleic acid binding resin to generate a purer preparation of DNA for subsequent transfection studies, and the use of a non-radioactive digoxigenin-tagged mitochondrial DNA probe. As shown in Fig. 1A, our modified conditions still produced results essentially identical to those previously published using a bolus of hydrogen peroxide [13, 14]; that is, the generation of significantly degraded mitochondrial DNA and the loss of the mature bands 3 h after initiation of hydrogen peroxide exposure (note: usually, two slow migrating bands are observed in DNA from unstressed cells, possibly representing supercoiled and relaxed forms of the circular mitochondrial genome).

In addition, studies were carried out to assess the effect of steady state hydrogen peroxide generation on mitochondrial DNA using glucose oxidase plus glucose (already present in the culture media). After initial titration, 7 and 20 mU/ml concentrations of glucose oxidase were selected for further study to determine the levels of hydrogen peroxide generated. As shown in Fig. 1B, sustainable hydrogen peroxide levels were generated at both glucose oxidase concentrations starting at 20 min through 5 h. Based on these results, we selected the 7 mU/ml glucose oxidase concentration, which generated 16 μM steady state hydrogen peroxide at 20 min and 1 h, and 15 μM at 5 h, as our test condition. Here, the cells were exposed to this level of glucose oxidase-generated hydrogen peroxide for 5 h. As shown in Fig. 1A right lane, significant

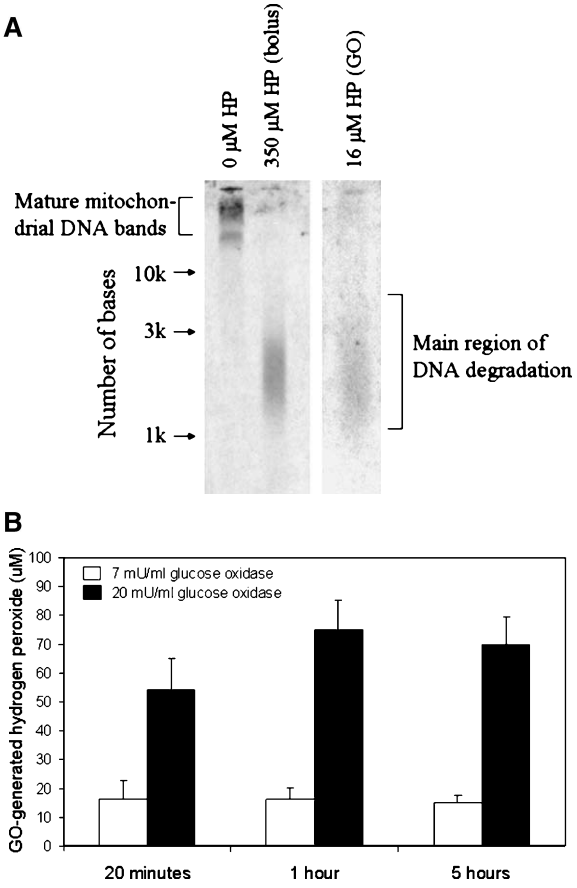

Fig. 1. Generation of oxidant-initiated degraded mitochondria DNA from HA-1 hamster fibroblasts. A) HA-1 cells were exposed to 0 and 350 μM hydrogen peroxide (HP) for 3 h, or 7 mU/ml of glucose oxidase for 5 h (16 μM steady state hydrogen peroxide), and at these times, cells removed from the incubator, spun and homogenized. Mitochondria were then isolated by sequential centrifugation, mitochondrial genomic DNA extracted and electrophoresed, transferred, and the Southern blot hybridized to the entire mitochondrial pAM1 genome labeled with digoxigenin as a probe. GO, glucose oxidase. B) Time course for the generation of hydrogen peroxide by 7 and 20 mU/ml glucose oxidase.

mitochondrial DNA degradation was observed here, and very similar to that observed for a 3 h bolus of 350 μM hydrogen peroxide (Fig. 1A, middle lane).

DeMP DNA is produced as early as 20 minutes following hydrogen peroxide exposure of HA-1 hamster fibroblasts

As shown above and previously reported under somewhat different conditions [13, 14], DeMP DNA can be generated in hamster HA-1 cells following 3 h of hydrogen peroxide exposure. We next considered whether DeMPs can be produced at earlier time points, since we previously showed that DeMP RNA is produced as early as 8–20 min following peroxide [12]. To determine how early DeMP DNA can be produced in cells, we exposed separate HA-1 cell cultures to peroxide for 2 h,1 h, and 20 min. As shown in Fig. 2, accelerated mitochondrial DNA degradation was observed as early as 20 min at both 150 and 350 μM hydrogen peroxide, with greater degradation at 1 and 2 h.

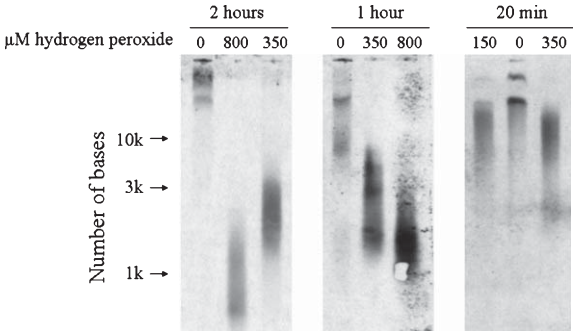

Fig. 2. The effects of hydrogen peroxide on the degradation of mitochondrial genomic DNA over time. HA-1 cells were exposed to various concentrations of hydrogen peroxide or solvent alone (control) as indicated for 2 h, 1 h, or 20 min, and at the appropriate time points, cells removed from the incubator, spun, and homogenized. Mitochondria were then isolated by sequential centrifugation and mitochondrial genomic DNA extracted, electrophoresed, transferred, and the Southern blot hybridized to the entire mitochondrial pAM1 genome labeled with digoxigenin as a probe.

Interestingly, the degradation of mitochondrial DNA as early as 20 min was similar to what we previously observed for RNA [12], suggesting that stress leads to a rapid and concomitant production of both DeMP DNA and RNA that may then act as danger signal alarmins.

Modest mitochondrial DNA oxidation is observed following hydrogen peroxide exposure of HA-1 hamster fibroblasts

To determine whether the observed mitochondrial DNA degradation is associated with oxidation, we assessed oxidation status using an antibody specific for oxidized guanine. This study was also valuable in better characterizing the nature of transfected DNA used for subsequent proinflammatory cytokine studies. Here, mitochondrial-enriched DNA from control and 2 h hydrogen peroxide-treated HA-1 cell cultures (using a 350 μM bolus) were compared by dot blot analysis. A 6.1-fold increase in oxidized guanine residues was observed in the enriched mitochondrial DNA from hydrogen peroxide-treated samples as compared with control (Fig. 3). Thus, the DNA used for subsequent proinflammatory cytokine studies (Figs. 4 and 5) was both degraded and modestly oxidized.

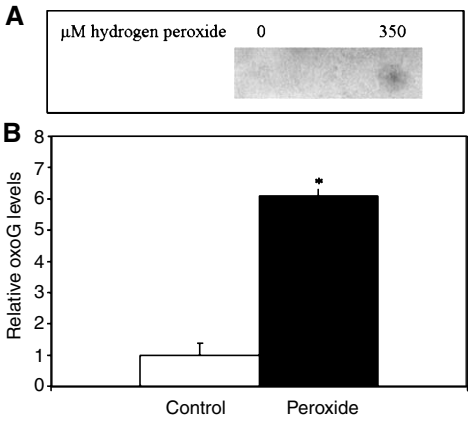

Fig. 3. The effects of hydrogen peroxide exposure on mitochondrial DNA oxidation status. HA-1 cells were exposed to solvent only (control) or 350 μM of hydrogen peroxide for 2 h, cells collected, and mitochondrial DNA extracted as described above. These DNAs were then dot blotted to nylon membrane, UV-irradiated, and hybridized with antibody specific to oxidized guanine (oxoG). A) Representative dot blot. B) Plotted values. Values represent means ($n = 3$) ± SEM, with the amount of oxidized mitochondrial DNA from control cultures normalized to 1.0. * Significant difference between the oxidation state of mitochondrial DNA from control versus hydrogen peroxide-treated cultures using a one-sample *T*-test at $p < 0.05$.

DeMP DNA induces a strong proinflammatory cytokine response in astrocytes

The ability of degraded mitochondrial DNA to act as an alarmin and elicit a proinflammatory response in astrocytes was assessed using transfection. Based on the above Fig. 2 results, we choose to used DeMP DNA from mitochondria obtained from cells treated for 2 h with 350 μM hydrogen peroxide for transfections, as this material was thoroughly degraded and of intermediate size (average 2500 bases) compared with DNA degraded with the higher 800 μM hydrogen peroxide concentration. Media collected 20 h later was then analyzed for levels of the released proinflammatory cytokines MCP-1, TNFα, and IL-6. Media from mock transfected cells was used as a control, while the common positive control Poly dAdT was also employed. As shown in Fig. 4, a strong proinflammatory response was observed in the cultured astrocytes. Specifically, degraded mitochondrial DNA induced MCP-1 levels 11.2-fold, and TNFα 8.4-fold, per the 0.4 μg of transfected DNA used for each culture well (Fig. 4A). These values actually exceeded that of the positive control dAdT DNA. The effect of the degraded mitochondrial DNA on IL-6 was especially dramatic (95-fold induction; Fig. 4B).

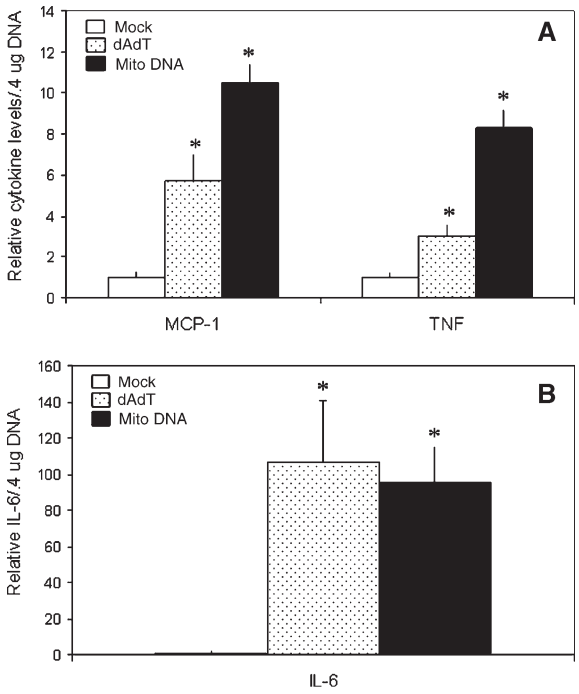

Fig. 4. DeMP DNA induces a strong proinflammatory cytokine response in astrocytes. DeMP DNA and control dAdT was transfected into mouse primary astrocytes and media collected 20 h later. Samples were then analyzed for MCP-1, TNFα, and IL-6 using the Cytometric Bead Array Flex Sets. Mock, no DNA mock transfected sample. Mock transfection values were normalized to 1.0. Values represent means ($n = 4$) ± SEM. * Significant difference between Mock and DNA transfected using a one-sample *T*-test at $p < 0.05$.

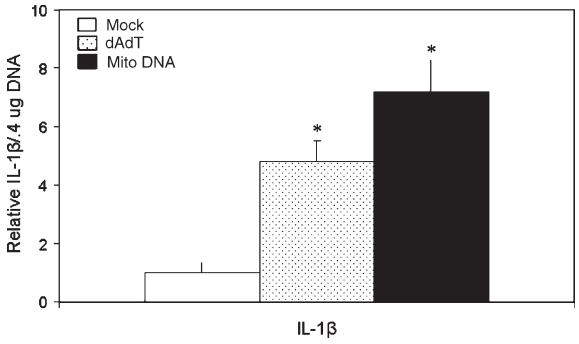

Fig. 5. DeMP DNA induces the inflammasome product IL1β in astrocytes. DeMP DNA and control dAdT were transfected into mouse primary astrocytes after priming 5 h with 200 ng/ml LPS, and media collected 20 h later. Samples were then analyzed for IL1β using the Cytometric Bead Array Flex Set. Mock, no DNA mock transfected sample. Mock transfection values were normalized to 1.0. Values represent means ($n = 3$) ± SEM. * Significant difference between Mock and DNA transfected using a one-sample *T*-test at $p < 0.05$.

Thus, DeMP DNA induces a strong proinflammatory cytokine response in cultured mouse primary astrocytes.

DeMP DNA also induces the inflammasome product IL1β in astrocytes

The inflammasone is a critical inflammatory response complex that mediates the production of IL1β and IL18 in immune cell types. Its presence and activity in other type cells including astrocytes is not well characterized. The above transfection experiment was therefore carried out and collected media analyzed for IL1β. For these studies, the astrocytes were first primed with 200 ng/ml LPS prior to transfection as described for other inflammasome studies [30, 32]. As shown in Fig. 5, the DeMP DNA significantly induced IL1β (7.2-fold). These results indicate that DeMP DNA can induce an inflammasome-mediated proinflammatory response. Combined with the Fig. 2 studies, to our knowledge, this is the first demonstration that astrocytes are capable of proinflammatory cytokine induction and inflammasome activation by any form of mitochondrial DNA.

DeMP DNA is detectable in human CSF and plasma

To determine whether degraded mitochondrial DNA is released into and present in brain fluid and blood, human CSF and plasma DNA was extracted and column purified. These DNAs were then electrophoresed, transferred to membrane, and the Southern blots probed with digoxigenin-tagged whole mitochondrial genome DNA. As shown in Fig. 6, detectable DeMP DNA was present in CSF and plasma, and present in a moderately degraded state using Fig. 1 as a guide (note: an interesting but as yet unclear variability in the degree of degradation between the two human plasma donors was observed). Thus, human CSF and blood plasma contain detectable DeMP signal.

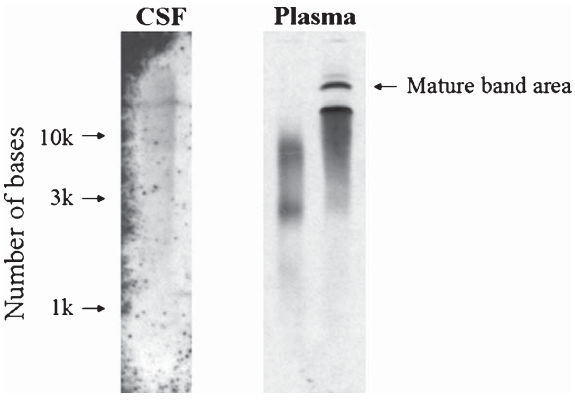

Fig. 6. DeMP DNA is detectable in human CSF and plasma. Human CSF and plasma were centrifuged, and the supernatant DNAs extracted and column purified. These DNAs were then electrophoresed, transferred to membrane, and the Southern blots probed with digoxigenin-tagged whole mitochondrial genome DNA.

DISCUSSION

Our present studies indicate that DeMP DNA, generated in a model of oxidative stress, is strongly immunogenic in astrocytes. These results provide new insights on a number of levels. For one, they demonstrate that a pathophysiologically relevant form of mitochondrial DNA (degraded and oxidized) can elicit a proinflammatory cytokine induction. As previously mentioned, other studies have demonstrated that mitochondrial DNA is able to stimulate induction of proinflammatory cytokines in immune cells, and that mitochondrial DNA can be detected in blood plasma. However, since PCR amplification has been used to assess this mitochondrial DNA amount, its actual structural state has never been assessed. In fact, these reports read as if it is assumed that released, proinflammatory mitochondrial DNA is the intact genome. This is unlikely since intact mitochondrial DNA is large and unlikely to readily pass through even semi-compromised mitochondria. Furthermore, we show here that mitochondrial DNA is present in CSF, and in a moderately degraded state. Finally, we also show that DeMPs are produced in HA-1 hamster fibroblasts in response to hydrogen peroxide exposure. This model mimics pathological states in that it is both a stress, and an oxidative one, and pathological states have often been associated with elevated ROS levels [1, 3, 5, 6]. In fact, hydrogen peroxide levels have been estimated as high as 100 μM (steady state) for certain pathological stress conditions including brain ischemia [41–44].

Our results also demonstrate, for the first time, that DeMPs can induce inflammasome activity. Inflammasome activity is a major contributor to inflammation and known to be activated by microbial and mitochondrial DNAs, in turn leading to the specific elevation and activation of IL-1 and IL-18 [19, 23, 29, 30, 32]. Our results, for the first time, also demonstrate that pathophysiologically-relevant DeMPs elicit a proinflammatory cytokine induction in a brain cell type (astrocytes). This induction of astrocyte proinflammatory cytokines has particular potential relevance in

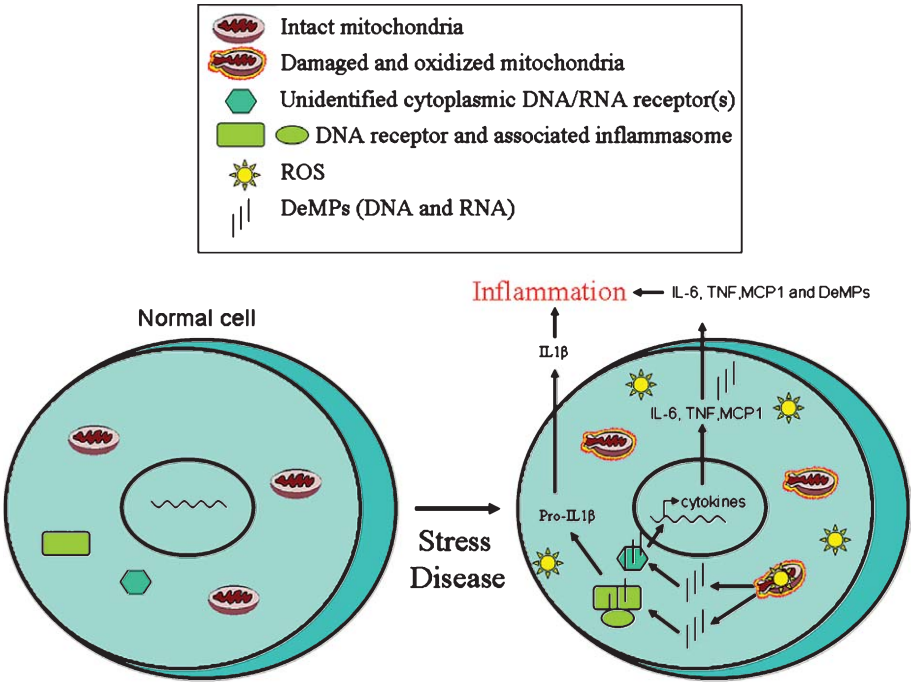

Fig. 7. Proposed model of DeMP release and signaling. During stress and the development of disease states, elevated ROS levels occur in cells and tissue. This elevated ROS oxidizes and damages cellular contents (in part due to elevated calcium levels) leading to compromised integrity of cell membranes including mitochondria. Additionally, as we have observed, degraded mitochondrial DNA and RNA is generated. These DeMPs then leak out into the cytoplasm and extracellular circulation. In the cytoplasm, they elevate proinflammatory cytokines which are then secreted, activating immune cells and inducing an overall organismal immune response that leads to inflammation. Circulating DeMPs may also exacerbate this inflammation state by externally triggering additional proinflammatory cytokine expression in immune and cytokine-producing cells.

neurodegenerative diseases as discussed below. As part of this, these results reveal, also for the first time, that astrocytes are capable of inflammasome activation by DeMPs.

Additionally, our results indicate that DeMPs are detectable in CSF and plasma, suggesting the importance of this structural form in mitochondrial DNA DAMP activity, and also demonstrating that cells and tissue are able to release this material. An elevation in CSF and plasma DeMPs may exist under pathological conditions, and future studies are planned to assess this such as by examining DeMP levels in CSF and plasma from Alzheimer's disease (AD) versus normal individuals. Other future studies will determine whether extracellular DeMPs can elicit a significant proinflammatory response in target cells (i.e., in the absence of transfection), and the effect of toll like- and DNA-receptor knockout mutants on this response to better assess the involved mechanisms behind DeMP pathobiology. Finally, our results demonstrate that hydrogen peroxide stimulates a parallel and early stage degradation of mitochondrial DNA and RNA, suggesting their role as early stage oxidative stress alarmins. We now also believe that our earlier studies going back to the late 1990 s represent some of the earliest reports of DAMPs, now that mitochondrial DNA has officially been found to be a DAMP family member, and since we have demonstrated that degraded mitochondrial DNA and RNA production is associated with growth arrest, apoptosis and adaptive response (then) and proinflammatory cytokine production (present studies).

For hydrogen peroxide from a bolus or glucose oxidase catalysis, or *in vivo* pathological pro-oxidant state formation from increased mitochondrial and NADPH ROS generation combined with reduced antioxidant status, the upstream signaling mechanisms would presumably be oxidative in nature leading to eventual DeMP generation. At the cell membrane, an oxidation of protein and lipids with subsequent lipid peroxide and aldehyde formation is likely and, in combination with permeabilized hydrogen peroxide, would lead to a plethora of cellular alterations contributing to DeMP formation. This would include released transition metals, altered protein signaling pathways (such as activation of redox-responsive MAP kinases), and the known release and intracellular elevation of

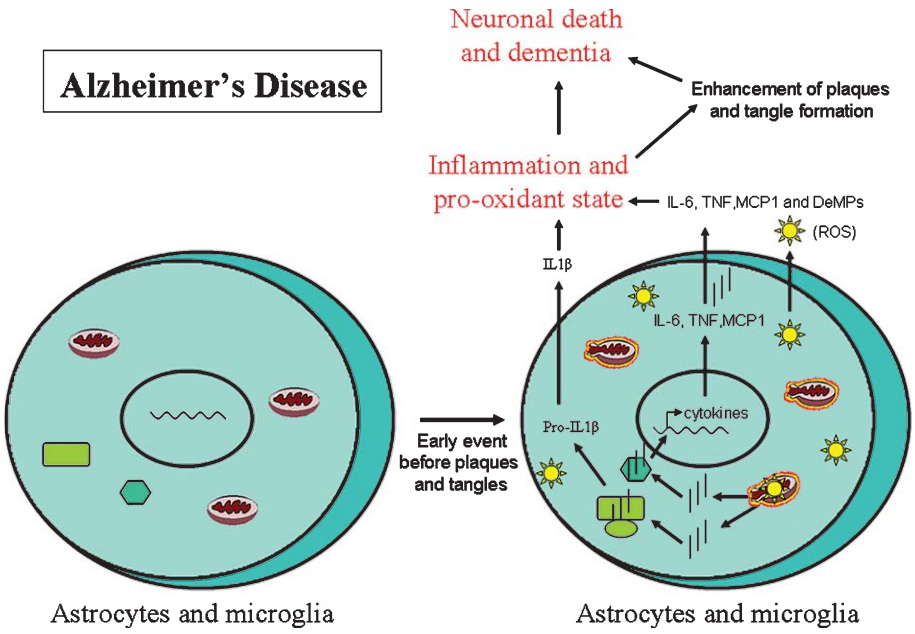

Fig. 8. Proposed model of DeMP involvement in Alzheimer's disease. In AD, elevated ROS occurs in astrocytes and microglia well before, perhaps decades before, the full-blown appearance of amyloid plaques and tau tangles. As outlined in Fig. 7, this eventually leads to DeMP release, then proinflammatory cytokine production, then brain inflammation and an even greater pro-oxidant state. This early and aberrant appearance of oxidative stress and inflammation eventually leads to neuronal death, perhaps in part by potentiating plaque and tangle formation.

free intracellular calcium from disrupted endoplasmic reticulum, mitochondrial, and plasma membranes [44]. We hypothesize that this elevated calcium activates nuclease(s) that target DNA and contributes to the observed DeMP generation [13]. Additionally, we observe a 6.1-fold increase in oxidized guanine residues in mitochondrial-enriched DNA from hydrogen peroxide-treated samples as compared with control (note: it is theoretically possible that there is some contaminant nuclear DNA in this preparation, so we refer to this DNA as "mitochondrial-enriched"). This increased oxidation is likely a minimal value, since greater oxidation may have occurred that was repaired during incubation. Thus, elevated ROS are also reaching the mitochondrial DNA in response to external hydrogen peroxide exposure. The main oxidizing species thought to be involved in DNA oxidation is hydroxyl radical [44], likely from the Fenton reaction at DNA in the presence of reduced transition metals that may also be elevated from the initial oxidative stress. Elevated superoxide from disrupted electron transport and NADPH activation, alkoxyl radicals, and lipid peroxyl radicals may also contribute, as well as toxic lipid peroxidation byproducts such as aldehydes that may promote mitochondrial DNA degradation.

In our HA-1 oxidative model, we observed DeMP DNA and RNA generation as reported here and previously [12–15]. Furthermore, we now demonstrate that the oxidant-initiated degraded form of DNA elicits a strong proinflammatory cytokine response in astrocytes. Combined, these results support a model in which the elevated ROS known to be associated with many pathological states triggers the generation of DeMPS, which are then released from ROS compromised/damaged mitochondria to the cytoplasm and extracellular circulation. This is further supported by the known release of mitochondrial DNA to the circulation in humans, and its elevation in pathological states [33–36]. Once outside of the mitochondria, these DeMPs are able to act as alarmins, inducing cytokine production in the case of cytokine producing cells including astrocytes, as well as other actions such as growth arrest, for which we previously demonstrated an association [14]. This DeMP effect may even be beneficial in some cases, as a protective "adaptive response" is also associated with DeMP formation as we have reported [12]. Based on our results and the above considerations, we propose two models as shown in Figs. 7 and 8. In the first, a general one, the effect of elevated ROS in pathological states is shown to trigger mitochondrial DNA and RNA degradation, which are then released to the cytoplasm and extracellular milieu and circulation through leaky compromised mitochondrial membranes. Here, they act as

alarmin danger signalers, and their effect includes the induction of proinflammatory cytokines in cytokine producing cell types, triggering an organismal immune response leading to inflammation. In the second, we consider the potential contribution of DeMPs to neurodegeneration using AD as a prime example based on our new astrocyte results. AD was discovered over 100 years ago but unfortunately, there is nothing close to a cure for this sad, debilitating and costly disease. While the overwhelming focus of AD research has been senile amyloid plaques and tau tangles, the lack of progress toward a cure has led an increasing number of researchers to consider the possibility that there are other bases for the disease. More recently, a so-called "Alternate Hypothesis" has been considered [45]. It states that although amyloid plaques are involved in the disease, they are not the initiating event and may be, in some cases, perhaps even be a protective brain response. We propose that DeMPs are a newly identified major contributor to AD. We base this claim on four things: 1) as we have shown, DeMPs can elicit a strong proinflammatory response in astrocytes; 2) inflammation, which would be increased by the DeMP elevated release of proinflammatory cytokines by astrocytes, is an early stage predictor of AD, where early and sometimes dramatic activation of cytokine overexpression occurs in astrocytes and microglia sometimes decades before pathological changes consistent with a diagnosis of AD are apparent [45–48]; 3) similar to inflammation, oxidative stress is also an early stage in AD [45, 48, 49], and our model uses ROS as a source to produce oxidized degraded mitochondrial polynucleotide alarmins; and 4) mitochondrial DNA already has a strong pathological connection, as highly elevated plasma levels have been associated with multiple diseases and disorders including brain [33–36].

In summary, we have extended previous studies and determined that oxidant-initiated DeMPs representing a likely pathophysiologically and *in vivo* relevant structural form is able to stimulate a strong proinflammatory cytokine induction of IL-6, MCP-1, and TNFα; that this proinflammatory effect also includes activation of inflammasome IL1β; that CSF and blood plasma contains detectable DeMP signal; and that early stage degradation of mitochondrial DNAs (and RNAs) in HA-1 hamster cells occurs following hydrogen peroxide exposure, suggesting their role as early stage alarmin signalers. We also show, for the first time, that this proinflammatory DeMP effect occurs in a brain cell type (astrocytes). Combined, these results provide new insights into the role and action of mitochondrial polynucleotides as DAMPs, and suggest a role for DeMPs in AD and other neurodegenerative and mitochondrial-based diseases.

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