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REVIEW

Aging: A mitochondrial DNA perspective, critical analysis and an update

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Abstract

The mitochondrial theory of aging, a mainstream theory of aging which once included accumulation of mitochondrial DNA (mtDNA) damage by reactive oxygen species (ROS) as its cornerstone, has been increasingly losing ground and is undergoing extensive revision due to its inability to explain a growing body of emerging data. Concurrently, the notion of the central role for mtDNA in the aging process is being met with increased skepticism. Our progress in understanding the processes of mtDNA maintenance, repair, damage, and degradation in response to damage has largely refuted the view of mtDNA as being particularly susceptible to ROS-mediated mutagenesis due to its lack of "protective" histones and reduced complement of available DNA repair pathways. Recent research on mitochondrial ROS production has led to the appreciation that mitochondria, even *in vitro*, produce much less ROS than previously thought, automatically leading to a decreased expectation of physiologically achievable levels of mtDNA damage. New evidence suggests that both experimentally induced oxidative stress and radiation therapy result in very low levels of mtDNA mutagenesis. Recent advances provide evidence against the existence of the "vicious" cycle of mtDNA damage and ROS production. Meta-studies reveal no longevity benefit of increased antioxidant defenses. Simultaneously, exciting new observations from both comparative biology and experimental systems indicate that increased ROS production and oxidative damage to cellular macromolecules, including mtDNA, can be associated with extended longevity. A novel paradigm suggests that increased ROS production in aging may be the result of adaptive signaling rather than a detrimental byproduct of normal respiration that drives aging. Here, we review issues pertaining to the role of mtDNA in aging.

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Key words: Mitochondrial DNA; Reactive oxygen species; DNA damage; DNA repair; Somatic mtDNA mutations; Antioxidants; Reactive oxygen species signaling; Mitochondrial DNA degradation; Electron transport; Aging

Core tip: The notion of reactive oxygen species (ROS) -mediated accumulation of mutations in mitochondrial DNA (mtDNA) as a driving force behind aging is increasingly losing ground forcing a revision of the Mitochondrial Theory of Aging. While mitochondrial involvement remains in the center of attention of aging research, the focus is shifting from mtDNA mutations to mitochondrial physiology. The positive effect of increased ROS production on longevity is increasingly viewed as evidence that increased ROS production in aging may be adaptive rather than maladaptive. This novel paradigm explains failure of antioxidants to delay aging in clinical trials.

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INTRODUCTION

While there is no universally accepted definition of the aging process, it is often defined as changes (mostly detrimental) that occur in organisms during their lifespan. Most researchers agree that aging is: (1) universal; (2) intrinsic (*i.e.*, "built-in"); (3) progressive; (4) deleterious; and (5) irreversible. The universality of the aging process suggests the existence of an equally universal mechanism or mechanisms that govern it. Over time, different aging theories proposed a variety of such basic mechanisms. Perhaps the most popular of these theories was (and, arguably, remains) the Free Radical/Mitochondrial Theory of Aging (henceforth MTA) first proposed by Harman[1] in 1956. Initially, this theory simply postulated that aging results from the accumulation of oxygen free radical [reactive oxygen species (ROS)] damage to cellular components, including nucleic acids[1]. Over the years, the theory was refined by, first, the identification of mitochondria as both the source and the target of the ROS[2], and, then, the identification of mitochondrial DNA (mtDNA) as a tally-keeper for the damage. The latter concept was introduced by Fleming *et al*[3] and Miquel *et al*[4,5], and is of particular importance because it provided an answer to critics who questioned the capability of other mitochondrial macromolecules such as lipids, proteins, or RNA to accumulate longitudinal damage over an organism's lifetime. Unlike damage to other macromolecules, damage to mtDNA can be converted to point mutations and deletions, which can be transmitted to and accumulated in daughter molecules through the process of replication, enabling deterioration of the integrity of hereditary information over time. It is this damage-sustaining capacity of mtDNA that makes it central to discussions of aging, and it is this property that will be the focus of the current review. Over the years, the MTA underwent many revisions to accommodate new experimental evidence, and thus, there are almost as many versions of it as there are investigators. As Jacobs observed more than a decade ago, "opponents of the hypothesis (MTA) tend to define it in such a narrow and extreme way that it is almost self-evidently falsified by generally accepted facts. Conversely, its proponents are liable to state the theory in such a vague and general way that it is virtually unfalsifiable experimentally"[6]. Here, we review our current knowledge of mtDNA maintenance as it pertains to the MTA, which consists of the following basic tenets: (1) Mitochondria are a significant source of ROS in the cell; (2) Mitochondrial ROS inflict damage on mtDNA; (3) Oxidative mtDNA damage results in mutations; (4) mtDNA mutations lead to the synthesis of defective polypeptide components of the electron transport chain (ETC); (5) Incorporation of these defective subunits into the ETC leads to a further increase in ROS production, initiating a "vicious" cycle of ROS production, mtDNA mutations, and mitochondrial dysfunction (Figure 1). This tenet appears to be the most controversial, and is no longer recognized as a part of the MTA by many researchers[7]; and (6) Eventually, mtDNA mutations, ROS production and cellular damage by ROS reach levels incompatible with life.

Some recent experimental evidence has called into question the validity of the MTA, prompting its reevaluation (see *e.g.*,[7]). Here, we present a historical perspective of our views on the role of mtDNA in aging and update our earlier critical review of the topic[8].

MTDNA

MtDNA (Figure 2) in mammals is a circular molecule that encodes 37 genes, including 2 rRNAs, 22 tRNAs, and 13 polypeptides. All 13 polypeptides are components of the oxidative phosphorylation (OXPHOS) system. They are encoded using a non-standard genetic code, which requires its own translational machinery separate from that of the nucleus. Two rRNAs and 22 tRNAs involved in this mitochondrial protein synthesis are also encoded by mtDNA. Mitochondrial DNA is densely packed into nucleoids, each containing as few as 1-2 mtDNA molecules[9].

A significant body of indirect evidence implicating mtDNA in longevity was contributed by studies on the inheritance patterns of longevity, which suggested possible cytoplasmic (mitochondrial) inheritance[10], and from studies which revealed the association of some mtDNA variations with longevity[11-14]. However, other studies indicate that these associations are weak[15]. The latest large-scale study on mtDNA and aging suggests that the relationship between mtDNA variants and longevity may be much more complex, and that while mutations in the OXPHOS complex I may beneficially affect longevity, the coincidence of mutations in complexes I and Ⅲ as well as the simultaneous presence of mutations in complexes I and V are detrimental. These more complex relationships escape detection by haplogroup analysis and require sequencing of complete mitochondrial genomes[16]. Overall, these findings indirectly support the idea that mtDNA variations may contribute to longevity.

MITOCHONDRIA ARE A SIGNIFICANT SOURCE OF ROS IN CELLS

ROS generation by mitochondria

In the course of their migration through the respiratory chain, electrons can "escape" and participate in the single-electron reduction of oxygen resulting in the formation of the superoxide radical ($O_2^{\bullet -}$ Eq. 1). The detailed overview of this process is presented elsewhere[8,17]. While the exact magnitude of ROS production *in vivo* re-

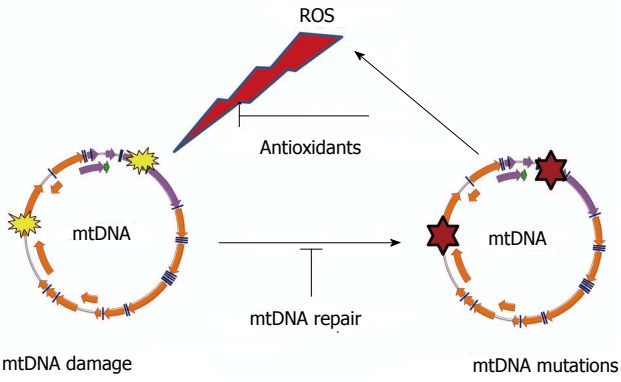

Figure 1 "Vicious cycle" of reactive oxygen species production, mitochondrial DNA damage, mitochondrial DNA mutagenesis and further reactive oxygen species production. The cycle implies an exponential growth of reactive oxygen species (ROS) production and mitochondrial DNA (mtDNA) mutagenesis.

mains debatable, we and others repeatedly argued[8,17] that the values of 1%-2% of total oxygen consumption[18] frequently cited in the literature are not reflective of physiological conditions and that the real rates are much lower.

ROS are produced by multiple sites in mitochondria[19]. Sites other than complexes I and Ⅲ are rarely mentioned in the context of aging. However, recent data suggest that some of these sites may have higher ROS production capacity than respiratory chain complex Ⅰ, which is often viewed as a major source of matrix superoxide production[20]. Moreover, it was argued that the endoplasmic reticulum and peroxisomes have a greater capacity to produce ROS than mitochondria do[21]. Another important consideration is that O_2•- produced by the mitochondrial respiratory chain inactivates aconitase, thus suppressing the Krebs cycle and reducing supply of NADH and $FADH_2$ to the respiratory chain. This can reduce electron flow through ETC, lower the reduction of ETC complexes, and diminish the production of O_2•-[22,23]. Thus, O_2•- production by ETC may be regulated by a negative feedback loop. Finally, actively respiring mitochondria may consume more ROS than they are capable of producing[24].

Mitochondrial ROS neutralization

ETC-generated ROS are detoxified through a two-step process. First, O_2•- is converted to H_2O_2 either spontaneously, or with the help of superoxide dismutases (Eq. 2). Two superoxide dismutases were described in mitochondria: SOD2 in the matrix and SOD1 in the intermembrane space. Interestingly, there is evidence of SOD1 activation by O_2•-[25]. The relative stability and membrane permeability of H_2O_2 ensure its ready access to mtDNA, yet like O_2•- this ROS is unable to efficiently react with DNA[8]. Only when H_2O_2 undergoes Fenton chemistry in the presence of transition metal ions (Eq. 3) is it converted to the extremely reactive hydroxyl radical. This ROS can efficiently damage mtDNA and other mitochondrial components[26,27]. At the second step, H_2O_2 in the mitochondrial matrix is detoxified by peroxiredoxins Ⅲ and Ⅴ (Prx Ⅲ and Prx Ⅴ, Eq. 4 and 5, respectively[28]) and by glutathione peroxidase 1 (GPx1, Eq. 6). Of the eight known GPx isoforms, this one is targeted to the mitochondrial matrix[29]. Another isoform, GPx4, is involved in detoxification of the mitochondrial membrane hydroperoxides[30] and is relevant due to the close association between mtDNA and the inner mitochondrial membrane. Prx Ⅲ is about 30-fold more abundant in mitochondria than GPx 1[31]. It is generally believed that catalase does not localize to mitochondria[32]. Therefore, GPx 1, and Prx Ⅲ and Ⅴ appear to be the main contributors to H_2O_2 detoxification in the mitochondrial matrix.

$O_2 + e^- \rightarrow O_2^{\bullet -}$ (Eq. 1)

$2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$ (Eq. 2)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\bullet}OH + OH^-$ (Eq. 3)

$H_2O_2 + 2\text{Prx Ⅲ}\ (SH)_2 \rightarrow 2H_2O + \text{Prx Ⅲ}\ (SH)\text{-S-S}(SH)\text{Prx Ⅲ}$ (Eq. 4)

$H_2O_2 + \text{Prx Ⅴ}\ (SH)_2 \rightarrow 2H_2O + \text{Prx Ⅴ}\ (S\text{-}S)$ (Eq. 5)

$H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2H_2O$ (Eq. 6)

Remarkably, the thioredoxin/peroxiredoxin system is capable of detoxifying extramitochondrial H_2O_2 in a respiration-dependent manner, providing evidence that mitochondrial OXPHOS is involved not only in the production of ROS, but also in their detoxification, and raising the question of whether mitochondria *in vivo* are a net source or a net sink of ROS[24].

MTDNA DAMAGE BY ROS

The reaction of O_2•- with non-radicals is spin forbidden[33-37]. In biological systems, this means that the main reactions of O_2•- are with itself (dismutation) or with another biological radical, such as nitric oxide. Therefore, direct reactions of O_2•- with mtDNA are unlikely. This ROS is far more likely to undergo dismutation to H_2O_2 (Eq. 2). As indicated above, H_2O_2 in the presence of transition metal ions, in particular Fe^{2+} and Cu^+, can undergo Fenton chemistry to form the extremely reactive •OH. Mitochondria are rich in iron, as many mitochondrial enzymes possess heme groups and iron-sulfur clusters in their active centers, and this abundance of iron may favor •OH production[38]. Therefore, it has been argued that mitochondria may be particularly susceptible to •OH -mediated oxidation, which plays a major role in DNA oxidation[39]. In this respect, it is important to note that mitochondrial iron is not free, but chelated (bound). Some experimental evidence does support the availability of chelated iron for Fenton-type reactions[40,41], and it is also true that iron chelators like desferrioxamine can efficiently suppress DNA mutagenesis by Fenton chemistry *in vitro*[42]. However, there is still a need for studies that could directly assess the ability of the iron bound in mitochondrial heme- and Fe-S proteins to promote generation of •OH.

Is mtDNA more sensitive to damage?

The mitochondrial genome accumulates germline mutations approximately one order of magnitude faster than

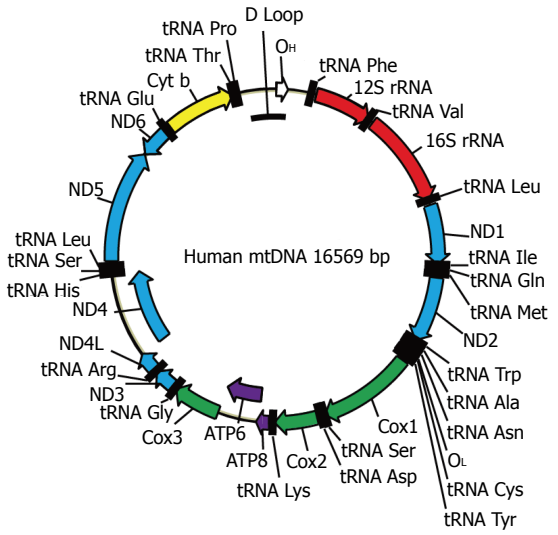

Figure 2 The map of human mitochondrial DNA. OH and OL: Origins of heavy and light strand replication, respectively; ND1-ND6: Subunits of NADH dehydrogenase (ETC complex I) subunits 1 through 6; COX1-COX3: Subunits of cytochrome oxidase subunits 1 through 3 (ETC complex IV); ATP6 and ATP8: Subunits 6 and 8 of mitochondrial ATPase (complex V); Cyt b: Cytochrome b (complex III); ETC: Electron transport chain.

nuclear DNA (nDNA)[43-45]. To evaluate relative accumulation of somatic mutations in nDNA *vs* mtDNA, we used 6×10^{-8} per nucleotide per cell division as an upper estimate for the rate of nDNA mutagenesis (8). Considering that the number of cells in the human body is 3.72×10^{13} (9), which roughly corresponds to 45 cell divisions starting with a fertilized egg, we arrive at $6 \times 45 \times 10^{-8} = 2.7 \times 10^{-6}$ mutations per base pair for the somatic nDNA mutation burden in an aged human, provided that there is no further nDNA mutagenesis after reaching adulthood. The somatic mtDNA mutation burden has been recently estimated to be 1.9×10^{-5} (10), which is less than 1 order of magnitude higher than the 2.7×10^{-6} just calculated for nDNA. mtDNA is turned over with half-lives of 10-30 d in different tissues (11), and therefore the difference in the rates of spontaneous somatic mtDNA mutagenesis between mtDNA and nDNA on per doubling basis may be even smaller than 1 order of magnitude [because in a 70-year-old human mtDNA has replicated on average (assuming a half-life of 30 d) at least $12/2 \times 70 + 45 = 465$ times compared to 45 times for nDNA, not counting repair synthesis]. Therefore, somatic mutations may accumulate at the same per doubling rate in nDNA as they do in mtDNA, while the cumulative burden of mutations in mtDNA may be one order of magnitude higher than that in nDNA in a 70-year-old individual.

In the literature, three properties of the mitochondrial genome are frequently cited as responsible for this faster rate of mtDNA mutagenesis: (1) Its proximity to the source of ROS (ETC); (2) Its lack of "protective" histones; and (3) A limited repertoire of DNA repair pathways available in mitochondria.

It has been argued, however, that proximity to the source of ROS, by itself, is unable to explain the higher mutation rate of mtDNA, and that some available experimental evidence directly contradicts the notion of the protective role of histones[8]. Observations that mtDNA is covered by TFAM[46] and that at least some prominent oxidative DNA lesions are repaired more efficiently in mitochondria than they are in the nucleus[47] also contradict the above arguments.

Moreover, mitochondria evolved a unique way to deal with excessive or irreparable damage: a pathway for degradation or abandonment of damaged molecules (Figure 3)[48,49]. This pathway is enabled by the high redundancy of mtDNA (hundreds to thousands of copies per cell). MtDNA degradation has been reported in response to both oxidative stress[50-52] and to enzymatically-induced abasic sites[53]. It also has been suggested that substrates for the Nucleotide Excision Repair pathway, which has not been detected in mitochondria, are also mitigated through mtDNA turnover[54,55].

If three of the above mentioned rationales in support of mtDNA's higher susceptibility to (oxidative) damage and mutagenesis are not satisfactorily supported by experimental evidence, what then is the basis for the frequently cited higher (compared to nDNA) susceptibility of mtDNA to oxidative stress? Here, one ought to make a distinction between damage to DNA bases-which may lead, upon replication, to point mutations-and damage to the sugar phosphate backbone. The first report comparing the content of the oxidative DNA base lesion, 8-oxodG, in nDNA *vs* mtDNA indicated that mtDNA may accumulate up to 15 times higher levels of this DNA oxidation product[56]. However, it was later established that this dramatic difference was a technical artefact[57]. Independent studies since confirmed that levels of 8-oxodG are similar in nDNA and mtDNA[58-60]. As far as sugar-phosphate backbone damage is concerned, Yakes and Van Houten[61] reported that in mouse embryonic fibroblasts exposed to H_2O_2, mtDNA accumulates more polymerase-blocking lesions than nDNA. These lesions are predominantly single- and double-strand breaks (SSB and DSB) as well as abasic sites with minor contribution from base modifications such as thymine glycol[50]. However, sugar-phosphate backbone damage may induce mtDNA turnover, thus preventing mutagenesis, rather than inducing it[48,50].

CAN MITOCHONDRIAL ROS INDUCE RELEVANT LEVELS OF MTDNA MUTATIONS?

Experimental evidence in support of the mutagenicity of mitochondrially produced ROS remains scarce. There are more studies attempting to assign oxidative stress as a cause of the observed mtDNA mutations than there are studies of mutations induced in mtDNA by experimental exposure of biological systems to oxidative stress. We were unable to detect a statistically significant increase in the level of mtDNA mutations in cells chronically treated with rotenone, which induces ROS production by inhibit-

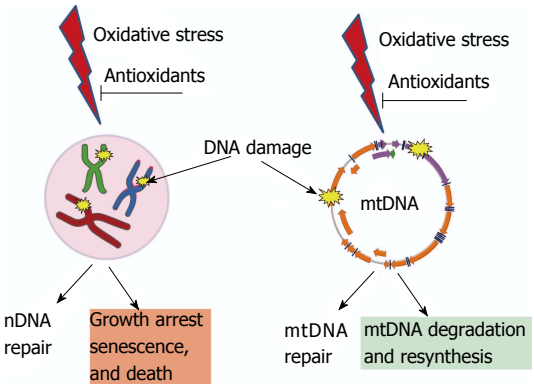

Figure 3 Consequences of unrepaired DNA damage in the nucleus and in the mitochondria. Oxidative damage induces lesions in both nDNA (left) and mtDNA (right). Both nuclei and mitochondria possess DNA repair systems to deal with these lesions. However, cellular consequences of unrepaired damage to nDNA and mtDNA are different. While persistent damage in nDNA results in the activation of cell cycle checkpoints, growth arrest, senescence and death. In contrast, mtDNA molecules with unrepairable damage are simply degraded and new molecules are synthesized using intact molecules as templates. This figure uses Servier elements available under Creative Commons license (155).

ing ETC complex I , and in cells repeatedly exposed to damaging levels of extracellular H_2O_2[50], which suggests that mtDNA is fairly resistant to ROS-induced mutagenesis. Similarly, recent studies indicate that mtDNA mutagenesis is not increased in flies with inactivated SOD and OGG1, an enzyme involved in the repair of oxidatively damaged DNA[62]. In aqueous environments, ionizing radiation induces DNA-damaging ROS: most importantly, the highly reactive •OH. With this in mind, Guo *et al*[63] evaluated 44 DNA blood samples from 18 mothers and 26 children. All mothers underwent radiation therapy for cancer in their childhood, and radiation doses to their ovaries were determined based on medical records and computational models. Sequencing of the entire mitochondrial genome in these patients revealed that the mother's age at sample collection was positively correlated with mtDNA heteroplasmy, a condition in which the cell possesses more than one mtDNA variant (the mitochondrial equivalent of nuclear heterozygosity). However, Guo *et al*[63] failed to detect any significant difference in single nucleotide polymorphisms between mother and offspring. Also, there was no significant correlation between radiation dose to the ovaries and the level of heteroplasmic mtDNA mutations among mothers and children. Therefore, radiation therapy-induced ROS do not appear to contribute, in a substantial way, to mtDNA mutagenesis[63]. This finding is significant because radiation therapy, by design, produces levels of ROS that are much higher than those observed under physiological conditions and therefore have a higher potential to overwhelm cellular antioxidant defenses and produce oxidative damage.

PROPERTIES OF AGING-ASSOCIATED MTDNA MUTATIONS

It is of note that even though age-associated mtDNA mutations are randomly distributed around the genome, there is some bias for the type of mtDNA mutations observed in aging in mitotic *vs* post-mitotic tissues. In mitotic tissues, most common type of mtDNA mutations identified is base substitutions. In contrast, large-scale-deletions are more commonly identified in post-mitotic tissues[64]. Among point mutations in dividing cells, transitions dominate the spectrum (90%) with the remaining fraction of mutations almost equally divided between transversions and small deletions. The frequency of non-synonymous (65.4%) and frameshift/premature termination codons (16.5%) in aging cells is significantly elevated as compared with variants found in the general population (34% and 0.6%, respectively). Also, the predicted pathogenicity of aging-associated mtDNA mutations is higher than that of mutations in the general population[64]. This suggests that human somatic cells, unlike germline cells[65], lack mechanisms to protect them from the accumulation of deleterious mutations.

The advent of Next Generation Sequencing enabled cost-effective interrogation of large numbers of mtDNA bases for mutations. These analyses revealed a minimal contribution of G > T transversions to the spectrum of aging-associated mutations. G > T transversions can be induced by 8-oxodG, a frequently used measure of oxidative DNA damage. This has led some investigators to conclude that oxidative damage does not contribute to aging-associated mtDNA mutagenesis[64,66]. Some observations, however, caution against this interpretation: (1) The most frequent base substitution induced by oxidative stress is a G > A transition[67,68]. This is the most prominent base change observed in mtDNA from aged tissues[66]; (2) 8-oxodG in mammalian cells can also induce G > A transitions[69], and therefore the available evidence does not allow for the complete exclusion of the contribution of this lesion to mtDNA mutagenesis in aging; (3) Cumulative evidence suggests that oxidative stress can induce all possible base substitutions, both *in vitro* and *in vivo*[68], cautioning against basing a conclusion regarding the involvement of oxidative stress in age-related mtDNA mutagenesis solely on an increase in the frequency of G > T transversions. Therefore, in the absence of studies that determine the mutational signature of ROS in mtDNA, any mutation can be interpreted as resulting from oxidative stress. And, conversely, no particular mtDNA mutation can be used, with confidence, as evidence of oxidative stress; (4) It has been shown that oxidative DNA damage does not necessarily lead to an increase in G > T transversions. For example, in DNA oxidatively damaged *in vitro* and passed through bacterial cells, the frequency of G > C transversions was increased, whereas the frequency of G > T transversions was actually decreased as compared to that of untreated DNA[70]. In an almost identical experiment, the frequency of base substitutions at A/T pairs in oxidatively damaged DNA was elevated, whereas the frequency G > T transversions remained unchanged after passing damaged DNA through mammalian cells[42]; and (5) The specific spectrum of oxidative-damage induced DNA mutations is determined, to a great extent, by the particu-

lar properties of the experimental system used (reviewed in[67]). At present, we lack a precise understanding of how oxidative mtDNA lesions are processed by mitochondria to produce mutations. Therefore, no definitive conclusion regarding the contribution of oxidative stress to the spectrum of aging-associated mtDNA mutations can be drawn from the absence of an increase in G > T transversions.

WHAT IS THE FUNCTIONAL SIGNIFICANCE OF AGING-ASSOCIATED MTDNA MUTATIONS?

Given that mtDNA mutations accumulate with aging, are they a cause of (1) mitochondrial dysfunction and/or (2) aging? It is well established that mitochondrial function is only compromised when the fraction of cellular copies of a given mtDNA-encoded gene affected by a given mutation exceeds a certain threshold specific to the mutation (and tissue). This threshold phenomenon can be mediated, at least in part, by intra- and intermitochondrial complementation[71-73]. It is usually accepted that this threshold is 60% to 70% of mutant mtDNA in chronic progressive external ophthalmoplegia and may be close to 95% in the syndromes of mitochondrial encephalopathy, myopathy, lactic acidosis, and stroke-like episodes, and myoclonic epilepsy with ragged red fibers[74]. Therefore, generally, more than 60% of cellular copies for a given mitochondrial gene have to be affected by a pathogenic mutation in order to observe phenotypic manifestation of the mutation[75]. In aging, mtDNA mutations are random, which brings about two caveats. First, not all aging-associated mutations are detrimental. Because of the degeneracy of the genetic code, 25% of mutations will not alter the amino acid sequence of the encoded protein (68.8% of mtDNA encodes for proteins), and others, while causing an amino acid or nucleotide substitution, will not negatively affect the function of the encoded protein or RNA molecule. Second, these mutations are not localized to a particular gene, but rather are randomly distributed among 37 mitochondrially-encoded genes. This means that in order to affect 60% of cellular copies of the largest mtDNA-encoded polypeptide MT-ND5 (which spans 11% of the mitochondrial genome), each mtDNA molecule has to carry on average 0.6/0.11 = 5.45 mutations. For smaller genes, this number will be proportionally higher. Since there is no experimental evidence that supports a selective advantage for deleterious point mutations, both of these caveats suggest that the presence of several aging-associated mutations per mtDNA molecule is required before impairment of mitochondrial function can be observed. These levels are indeed achieved in tissues of mtDNA mutator mice[76,77], but not in naturally aged tissues of experimental animals or humans. Based on the reported frequency of mtDNA mutations, it can be calculated that in mice aged 24-33 mo, mutations affect as little as 20% of mtDNA molecules[78]. Similar calculations using reported values for humans aged 75-99 years[66] suggest that only about 32% of mtDNA molecules are affected by mutations. Therefore, it is highly unlikely that the relatively low mutation loads observed in naturally aged tissues[50,79,80] can account for the observed age-related measurable decline in mitochondrial function and, by extension, cause aging, provided that these mutations are maintained in a heteroplasmic state. Intriguingly, though, some studies indicate that the fraction of respiratory chain-deficient colonocytes in aging mammalian tissues increases after 35 years, and by 70 years of age, up to a third of colonocytes can be respiration-negative. This can be explained by a random genetic drift model. According to this model, multiple rounds of replication may result in the clonal expansion of random mtDNA molecules, leading to a loss of heteroplasmy[81]. In humans, this model predicts that clonal expansion may take decades to occur. Therefore, random drift may provide a satisfactory explanation for the mechanism of respiratory dysfunction observed in aged tissues provided that it can be demonstrated that cell types other than colon epithelium accumulate similar levels of clonally expanded mutations. The random genetic drift in colon epithelium, the tissue in which this phenomenon is best understood, however, appears to be highly heterogeneous, and its extent does not correlate well with chronological age between individuals. For example, a 75-year-old individual may have a lower percentage of respiration-deficient crypts than a 45-year-old[82]. This heterogeneity is inconsistent with the steady and relatively uniform process of aging, and, therefore, argues against random genetic drift being the sole or even a major driving force of aging. It is also unclear whether clonally expanded somatic mtDNA mutations can drive aging in short-lived species. For example, in human colon such mutations are not detectable until about 30 years of age[82]. Can clonally expanded mtDNA mutations explain aging in *Caenorhabditis*. elegans whose lifespan is only 2-3 wk? It is implausible that mtDNA in this organism turns over so much faster to allow for clonal expansion comparable to that observed in humans. Therefore, clonally expanded mtDNA mutations are more likely to be a contributing, rather than driving, factor of aging.

IS THERE EVIDENCE FOR THE EXISTENCE OF THE "VICIOUS" CYCLE?

As noted above, "vicious" cycle is the most contentious part of the MTA. The main premise of the "vicious" cycle hypothesis is the existence of a feed-forward cycle of ROS production and mtDNA mutation. That is: (1) increased ROS production in aging leads to increased mtDNA mutagenesis; and (2) increased mtDNA mutation loads result in increased mitochondrial dysfunction and ROS production. The first part of this premise appears intuitive and plausible. Indeed, no antioxidant defense or DNA repair system works with 100% efficiency, and an increase in ROS will inevitably lead to an increase in mtDNA damage and mutagenesis, however little. The

second part of this premise, however, is more contentious. While observations in patients with mitochondrial disease may partially support the notion of increased ROS production in response to increased mtDNA mutation loads, these observations, paradoxically, also refute this notion. First, while some pathogenic mtDNA mutations result in increased ROS production[83,84], this is not a universal property of mutations in mtDNA. This point is best illustrated by observations made in "mito-mice" (mice that age prematurely due accumulation of random mtDNA mutations): these mice accumulate mtDNA mutation loads exceeding those observed in normal aging by more than one order of magnitude, and still this increase does not result in elevated levels of ROS production[76,77,85]. Thus, the majority of mtDNA point mutations will not affect mitochondrial ROS production regardless of their levels. Second, no accelerated aging or increase in mtDNA mutagenesis rates were reported in patients with mitochondrial diseases which are characterized by increased ROS production. Therefore, while increased ROS production is expected to increase the rate of mtDNA mutagenesis, this increase may not be physiologically relevant or experimentally detectable. This second point is relevant to the discussion above regarding threshold levels of mtDNA mutations.

Moraes *et al*[42] argued that if a "vicious" cycle played an important role in the accumulation of mtDNA deletions in somatic tissues, patients with compromised OXPHOS should accumulate mtDNA deletions at an accelerated rate. Their experiments did not support this prediction, leading Moraes *et al*[42] to the conclusion that a "vicious" cycle is not likely to play an important role in the accumulation of age-related mtDNA deletions[86].

To reconcile MTA with the new evidence, Gustavo Barja has put forward a new version of it that does not include the "vicious" cycle. Barja argues that the damage amplification step provided by the "vicious" cycle is unnecessary for the validity of the MTA[7].

ROS PRODUCTION AND LONGEVITY

It is predicted by the MTA that higher ROS production should lead to increased cellular oxidative stress, which should result in increased damage to cellular macromolecules including mtDNA, and ultimately lead to reduced longevity. Conversely, all other conditions being equal, lower ROS production and oxidative stress are expected to be associated with increased longevity. Since the principal contribution of the mtDNA to the aging process, within the framework of the MTA, is through the effects of mtDNA instability on cellular ROS production, it follows that an examination of the role of ROS in aging would be informative. Indeed, the lack of unequivocal evidence establishing a causative role for ROS in aging makes alterations in mtDNA, which are purportedly induced by ROS and contribute to aging by increasing ROS production, irrelevant.

Evidence from animal models

Early on, comparative biology studies established a positive correlation between body size and longevity. More detailed biochemical studies revealed an inverse correlation between mitochondrial ROS production and mtDNA damage on one hand and longevity on the other, across different biological taxa (reviewed in ref[7]), which is in agreement with the MTA. Unexpectedly, and conflicting with the predictions of the MTA, antioxidant defenses also correlated negatively with longevity[87]. Perhaps not surprisingly, an extension of this analysis to other species revealed that in many species, long lifespans defied explanation by the tenets of the MTA. One of the most striking examples in this category is that of the naked mole-rat. These animals, about the size of mice, live almost 8 times longer than mice[88,89]. Strikingly, these animals have very unremarkable antioxidant defenses: their glutathione peroxidase levels are 70 times lower than in mice, resembling those of knockout animals[88]. In the absence of compensatory upregulation of other antioxidant systems, this, predictably, leads to higher levels of oxidative damage in these animals: at least 10-fold higher levels of urinary isoprostanes (a marker of oxidative stress), eightfold increased levels of 8-oxodG (increased DNA damage) in the liver accompanied by reduced urinary excretion of 8-oxodG (reduced DNA repair), and high cellular (especially, mitochondrial) protein carbonyls were reported in this study[89]. The fact that naked mole-rats live longer than mice despite this increased oxidative burden (especially in mtDNA and mitochondrial proteins) strongly argues against the role of oxidative damage as a key determinant of longevity.

Another line of evidence against the MTA comes from studies on C. elegans. This organism has five genes encoding different isoforms of the SOD, an enzyme catalyzing the first step in the detoxification of superoxide (Eq. 2). Inactivation of the SOD isoforms in this organism either individually or in groups of three (including inactivation of all mitochondrial isoforms), failed to decrease the lifespan[90]. Instead, inactivation of *sod-2* led to increased longevity, which was associated with increased oxidative damage to proteins. Moreover, an *sod-2* mutation further increased lifespan of long-lived clk-1 mutants. Finally, the same group has recently inactivated all five sod genes in C. elegans and demonstrated that while animals completely lacking any SOD activity are more sensitive to multiple stressors, they have normal longevity[91]. Similarly, inactivation of the major mitochondrial antioxidant system by mutating Prx Ⅲ (Eq. 4) decreased overall fitness in this organism, but failed to affect the lifespan[92].

In the fruit fly, somatic mtDNA mutagenesis was not affected by inactivation of SOD either alone, or in combination with OGG1, an enzyme involved in repair of oxidative DNA damage, even though lifespan was affected[62]. These observations suggest a minimal contribution of oxidative stress to age-related somatic mtDNA mutagenesis.

Mclk1$^{+/-}$ mice heterozygous for the key enzyme in the biosynthesis of ubiquinone, an electron transporter

and mitochondrial membrane antioxidant, demonstrate extended longevity. This genetic defect is accompanied by an impairment of the ETC and by increased mitochondrial, but not cytoplasmic, oxidative stress[93]. Inactivation of the homologous gene *clk-1* in C. elegans also resulted in increased longevity. This led the authors to hypothesize that an increase in the generation of mitochondrial ROS might accompany aging not because ROS play a causal role in this process but rather because ROS stimulate protective and restorative processes that help to counteract age-dependent damage[94,95].

Track record of antioxidant-based life-extending strategies

It is predicted by the MTA that reducing intracellular ROS production should reduce damage to macromolecules, including mtDNA, and ultimately increase longevity. As a result, numerous interventional studies have been performed in both vertebrate and invertebrate models. Treatments in these studies typically included either lifelong supplementation with nonenzymatic antioxidants or genetic manipulation of intracellular levels of enzymatic antioxidants. These studies produced inconclusive results: while in some instances it was possible to achieve a modest increase in longevity, many studies revealed the lack of correlation, or even a negative correlation, between antioxidant defenses and lifespan (reviewed in ref[7]). In some instances, these studies produced different results in different species. For example, mitochondrial expression of catalase was reported to have no effect on the longevity of drosophila[96], but resulted in a modest (17%-21%) lifespan extension in mice[97]. In contrast, in C. elegans, a fivefold increase in longevity was reported for animals carrying two mutations (*daf-2 and clk-1*) in nDNA[98]. This suggests that nuclear genes play a pivotal role in determining longevity. To date, no manipulation of mtDNA or the systems involved in its replication, maintenance, or repair has produced comparable extension of the lifespan.

Howes[99] reviewed the results of antioxidant studies which involved more than 550000 human subjects, and concluded that "not only have antioxidants failed to stop disease and aging but also they may cause harm and mortality, which precipitated the stoppage of several large studies". Recent meta-studies support his findings: Bjelakovic *et al*[100] analyzed the results of 78 studies between 1977 and 2012, involving a total of 296707 participants, and concluded that antioxidant supplements neither reduce all-cause mortality nor extend lifespan, while some of them, such as beta carotene, vitamin E, and higher doses of vitamin A, may actually increase mortality[100]. The most direct interpretation of these findings in the context of the MTA as it pertains to mtDNA is that reduced oxidative damage to mtDNA does not extend longevity.

Caloric restriction (30%-40% reduction in caloric food intake without malnutrition) is frequently cited as the most reliable means of extending lifespan across diverse taxa and is frequently employed as a means to investigate the mechanisms of aging. Its effect is widely attributed to reduced ROS production and mtDNA damage[101]. However, in a recent survey of 41 laboratory mouse strains, 40% caloric restriction shortened lifespan in more strains than in which it lengthened it[102]. Similarly, a recent study by the National Institute of Aging revealed no beneficial effect of caloric restriction on longevity in primates[103,104].

CONCLUSION

Recently, there has been an emergence of experimental data challenging many aspects of the MTA as defined in the Introduction. This, in turn, has resulted in both a growing skepticism towards the role of mtDNA mutations in aging, and in the transformation of some of our views on mtDNA, ROS, and aging. Thus, the increased susceptibility of mtDNA to ROS-induced strand breaks (but not to oxidative base damage) is now viewed as a component of the mitochondria-specific mechanism for the maintenance of mtDNA integrity through abandonment and degradation of severely damaged mtDNA molecules, rather than as a mechanism for accelerated mtDNA mutagenesis (Figure 3). Also, we have begun to appreciate that increased ROS production in aging may represent evidence for adaptive signaling aimed at mitigating detrimental changes, rather than constituting an unwanted but unavoidable byproduct of respiration.

Even though its current status is controversial, it is the MTA that stimulated the research that advanced our understanding of aging and clarified the place of mtDNA in this process. While it is no longer plausible that mtDNA is either the sole or the main determinant of aging, epidemiological studies do still suggest a contribution of mtDNA variation to longevity[16]. Also, it is becoming increasingly obvious that maternally transmitted low levels of germline mtDNA mutations can have a significant impact on health and lifespan[105]. The random genetic drift theory[81] has the potential to reconcile the observed mitochondrial dysfunction in aged organs with the low average levels of mtDNA mutations in some tissues. These and other findings demonstrate that despite dramatic advances, our understanding of the role of mtDNA in aging remains incomplete. This incomplete understanding persists in large part due to our limited ability to manipulate mitochondria in a meaningful way. The lack of approaches to introduce defined base lesions into mtDNA impedes our progress in understanding the specifics of mitochondrial processing of oxidative DNA damage. This, in turn, limits our ability to deconvolute and interpret the spectrum of mtDNA mutations observed in aging.

In the near future there is great promise for further advances in our understanding of mtDNA's contribution to aging. The advent of Duplex Sequencing methodology now makes it possible to determine the mutational signature of oxidative stress in mitochondria, which is one

of the most important next steps in mtDNA research. The dire need for reliable markers of oxidative mtDNA damage is becoming increasingly obvious. Despite concerted efforts[106,107], detection of the widely used marker 8-oxodG remains variable between labs, which has resulted in contradictory reports: both a 20-fold increase[108] and no change[109] in 8-oxodG content in the mtDNA of OGG1 knockout animals have been reported. The development of methods for the determination of both the identity of mitochondrial ROS generated in vivo and the rates of their production would greatly aid in evaluating the interactions between mtDNA and ROS. Finally, a better understanding of the incidence, kinetics, and extent of random intracellular drift of mtDNA heteroplasmy in different tissues is needed for an accurate determination of its possible contribution to mitochondrial dysfunction in aging.

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REVIEW

Combinations of vascular endothelial growth factor pathway inhibitors with metronomic chemotherapy: Rational and current status

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Abstract

Chemotherapy given in a metronomic manner can be administered with less adverse effects which are common with conventional schedules such as myelotoxicity and gastrointestinal toxicity and thus may be appropriate for older patients and patients with decreased performance status. Efficacy has been observed in several settings. An opportunity to improve the efficacy of metronomic schedules without significantly increasing toxicity presents with the addition of anti-angiogenic targeted treatments. These combinations rational stems from the understanding of the importance of angiogenesis in the mechanism of action of metronomic chemotherapy which may be augmented by specific targeting of the vascular endothelial growth factor (VEGF) pathway by antibodies or small tyrosine kinase inhibitors. Combinations of metronomic chemotherapy schedules with VEGF pathway targeting drugs will be discussed in this paper.

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Key words: Metronomic chemotherapy; Vascular endothelial growth factor; Angiogenesis; Bevacizumab; Vascular endothelial growth factor receptor

Core tip: Metronomic chemotherapy has the potential to reduce the toxicity of chemotherapy administered with conventional schedules. In addition, understanding of the importance of angiogenesis in the mechanism of action of metronomic schedules provides a rational to combine this type of administration with targeted agents against the vascular endothelial growth factor signaling pathway.

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INTRODUCTION

Metronomic chemotherapy is defined as a chemotherapy treatment that is given more often but in lower doses than conventional chemotherapy[1]. The different administration schedule results in differences in pharmacokinetics of the given drugs and in general lower peak concentrations but more protracted trough concentrations. These pharmacokinetic differences may have implications for anti-tumor efficacy but equally importantly for adverse effects and tolerability of chemotherapy drugs.

Vascular endothelial growth factor receptors (VEGFRs) are a family of tyrosine kinase cell surface receptors that includes VEGFR-1 (also called Flt-1), VEGFR-2 (also called KDR), VEGFR-3 and the two neuropilin co-receptors NRP-1 and NRP-2. These are ligated by six secreted glucoprotein ligands VEGF-A to -E and PIGF

(Placenta Growth Factor). Ligation of the receptors triggers activation of down-stream cascades that include the Ras-Raf-MAPK and the PI3K-Akt pathways. VEGFR signaling leads to loosening of the inter-cellular junctions of endothelial cells and contributes to increased motility and eventually results in promotion of angiogenesis and vascular permeability[2]. The VEGFR system has a physiologic role during development that is usurped by tumors. Hypoxia in the tumor micro-environment is a well-known inducer of VEGF. Its expression is up-regulated by transcription factor [hypoxia-induced factor (HIF)], a factor consisting of two sub-units HIF-1α or HIF-2α and HIF-β. The β sub-unit is constitutively expressed and the α sub-units are up-regulated by hypoxia through a mechanism involving hypoxia-promoted protein stabilization. Hypoxia prevents proline hydroxylation of HIF-α factors interfering with their interaction with ubiquitin ligase (Von Hippel Lindau) and thus prevents them from being ubiquitinated and degraded in the proteasome[3]. As a result HIF-α factors concentration is increased and in association with HIF-β may initiate transcription of more than a hundred target genes, such as VEGF[4]. Other tumor associated events, such as tumor suppressor p53 inactivation and proliferation-promoting cascades activation, induce VEGF.

In this paper we will review the current data on combinations of VEGF and VEGFR inhibitors with metronomic chemotherapeutics in cancer. Drugs that affect other components of the angiogenesis network or inhibit angiogenesis indirectly may constitute rational partners for development in combination with metronomic chemotherapy but will not be discussed here.

COMMONLY USED METRONOMIC CHEMOTHERAPIES

Metronomic chemotherapy that comprises the backbone of combinations with anti-angiogenic agents is most often given in an oral form and thus availability of an oral form of various chemotherapeutics has dictated the development of such combinations, given the considerable greater ease and practicability of administering lower closely spaced in time (*e.g.,* daily) doses by mouth instead of intravenously. All these oral drugs have been developed as monotherapies or combinations with classic chemotherapy drugs previously and have a well-characterized safety profile when used in conventional doses and schedules. Data also exist for metronomic schedules, although in general less extensive than for conventional schedules. Some intravenous (IV) chemotherapy drugs given in lower doses and more frequently schedules, usually weekly, instead of three-weekly, can be considered metronomic.

Most commonly used oral chemotherapies include oral forms of cyclophosphamide, methotrexate, vinorelbine, etoposide and topotecan, the oral alkylator temozolamide and the oral fluoropyrimidines capecitabine and tegafur in combination with uracil. Metronomic oral cyclophosphamide is most commonly dosed at 50 mg per day instead of the more conventional dose of 100 mg/m^2 per day for 14 d every 4 wk incorporated, for example, in one of the versions of CMF and 600 mg/m^2 IV every 3 wk in another CMF version[5,6]. Metronomic oral methotrexate has been used often in combination with metronomic cyclophosphamide in metastatic breast cancer at a dose of 2.5 mg twice weekly as compared with the dose of 40 mg/m^2 of the classic CMF (of note this dose is considered "low" compared to "intermediate" and "high" doses in the grams range used in the treatment of sarcomas and acute leukemias and requiring folinic acid rescue)[7,8]. A usual dose of oral daily etoposide is 50 mg and a proposed dose of daily oral topotecan is 0.8 mg/m^2[9]. A commonly used metronomic dose of temozolomide is 75 mg/m^2 continuously daily compared with 150-200 mg/m^2 for 5 consecutive days every 4 wk that is a more conventional dose[10]. Of interest, the metronomic administration is part of a first line standard regimen in glioblastoma multiforme in combination with radiation therapy[10].

Metronomic administration of chemotherapy has essentially a lower incidence of some of the most serious and problematic adverse effects of conventional doses of chemotherapeutics such as myelotoxicity and serious gastrointestinal toxicity. In many cases metronomic chemotherapy produces significantly less alopecia that, although not life-threatening, is impacting in patients' quality of life.

TARGETED AGENTS AGAINST VEGF AND VEGFRS

The first anti-angiogenic agent targeting the VEGF-A ligand that entered the clinical arena was the recombinant humanized monoclonal antibody bevacizumab. Activity has been shown mainly in combination with classic chemotherapy agents in a variety of tumors such as colorectal cancer, glioblastoma multiforme, ovarian cancer and carcinomas of the lung[11-14]. Not surprisingly bevacizumab is the agent targeting the VEGF/ VEGFR axis most extensively studied also in combination with metronomic chemotherapy.

An agent targeting the VEGF ligand, that was more recently developed, is the VEGF trap aflibercept. This molecule consists of the second immunoglobulin domain of VEGFR-1, the third immunoglobulin domain of VEGFR-2 and the Fc (constant region) of IgG1 type human immunoglobulins. The construct functions as a decoy receptor that captures circulating VEGF-A and PlGF, preventing them from binding and activating their receptors[15]. Aflibercept has been approved for the treatment of metastatic colorectal carcinoma in combination with the FOLFIRI regimen[16] but has not been studied clinically in combination with metronomic schedules.

An increasing number of small molecules tyrosine kinase inhibitors (TKIs) blocking VEGFRs have gained approval for diverse indications and have entered the

clinic. They are less specific than monoclonal antibodies and tend to block other tyrosine kinase receptors and even non-receptor kinases with varying degrees of affinity. The TKI regorafenib, for example, that is approved for the third line treatment of metastatic colorectal cancer, inhibits, in addition to VEGFR, PDGF, FGFR, C-kit, Ret, BRAF and TIE-2[17]. TKIs sunitinib and pazopanib, approved for the treatment of renal cell carcinoma inhibit, in addition to VEGFR, PDGFR and C-kit, while sorafenib, another TKI also approved for the treatment of renal cell carcinoma as well as for hepatocellular carcinoma, inhibits, in addition to these three receptors, non-receptor kinase BRAF[18].

Targeted agents have a different toxicity profile from chemotherapy in general and metronomic administration in particular. A side effect peculiar to all VEGF pathway blocking agents is hypertension[19]. Skin reactions, hypothyroidism and blood coagulation complications are also other adverse effects.

RATIONAL FOR THE COMBINATION

Metronomic chemotherapy is believed to act against cancer cells in an indirect way through anti-angiogenic actions as well as through immune-mediated effects. An experimental demonstration of the importance of angiogenesis for tumor propagation in mice was obtained by using low dose metronomic cyclophosphamide. In these classic experiments metronomic cyclophosphamide inhibited angiogenesis *in vivo* and induced apoptosis of endothelial cells in a mouse experimental model[20]. This was followed by apoptosis of the human drug-resistant leukemia and lung cancer cell xenografts. Combined treatment of mice with metronomic cyclophosphamide and an angiogenesis inhibitor, TNP-470, resulted in complete eradication of drug-resistant human xenografts. Metronomic schedule consisted of cyclophosphamide 170 mg/kg every 6 d while the classic dose was 150 mg/kg in days 1, 3 and 5 every 3 wk. The effect of metronomic doses of vinblastine has been studied in human umbilical vein endothelial cells (HUVEC). Doses of 0.25-1 pM of the drug produced significant decrease in the angiogenic phenotype (proliferation, chemotaxis and adhesion to extracellular matrix components) without an increase in apoptosis of HUVEC[21]. These experiments argue for the relevance of angiogenesis in the effects of metronomic chemotherapy schedules and, in addition, offer the first evidence for the value of adding a targeted angiogenesis inhibitor.

An additional mechanism of anti-tumor action of metronomic chemotherapy is promotion of the immune response against cancer cells. In a model of tumor bearing mice, metronomic paclitaxel was demonstrated to enhance immune responses obtained with immunization with a DNA vaccine against chimeric CTGF/E7[22]. Another study of metronomic cyclophosphamide in mice bearing xenografts of glial origin or syngeneic tumors confirmed an anti-tumor activity that was dependent on recruitment of immune cells in the tumor[23]. Mice with a severe combined immunodeficiency background or defects on perforin could not mount an anti-tumor response to metronomic cyclophosphamide. In addition treatment with a more conventional intermittent schedule of cyclophosphamide produced a weaker and transient immune activation, a fact interpreted by the authors to denote a need for a more sustained immune stimulation for effective immune response production[23]. Interestingly, in this study, combined treatment with inhibitors of VEGFR axitinib, cediranib and AG-028262 and metronomic cyclophosphamide interfered with the ability of the latter to recruit immune cells to the tumors and decreased the response to it. Immune mediated activity of metronomic chemotherapy appears to be particularly dependent on doses and frequency of the used drugs, at least in pre-clinical mouse models[24]. Work from the same laboratory has shown that, in contrast to immune interference, anti-VEGFR agents may have synergistic anti-tumor effects with metronomic chemotherapy by promoting tumor retention of active metabolites such as 4-hydroxy-cyclophosphamide and, at least partially counteracting, the deleterious effect on anti-tumor immunogenicity[25]. In discordance with the above pre-clinical data arguing for a role of metronomic schedules in triggering anti-tumor immunity, a small clinical study of patients with diverse types of cancer treated with conventional or metronomic schedules of chemotherapy has reported an increase in the ratio of regulatory T cells to effector T cells in both types of schedules but this increase was more pronounced with metronomic schedules[26]. Regulatory T cells may blunt the anti-tumor response of the immune system against tumor cells. The study did not aim to compare the implications of these immune effects on clinical outcomes and, in any case, this would be impossible given the small number of patients across different tumor types and different drugs used[26]. Overall the involvement of an immune response to the anti-tumor effect of metronomic schedules of chemotherapy is far from clear but the addition of VEGF pathway-targeting agents could theoretically improve this immune effect by normalizing the tumor vasculature network and thus improving immune cell access to the tumor by preventing the formation of the pathologic tumor-associated convoluted vessel network. This is due to the fact that morphologically abnormal glomeruloid microvascular proliferations and bridged mother vessels remain dependent on VEGF-A signaling after their formation while feeder arteries, draining veins and capillaries in the tumor beds become VEGF signaling-independent[27].

PRE-CLINICAL EVALUATION

Consolidating the above rational, additional pre-clinical evaluation of combinations of metronomic chemotherapies with anti-VEGF targeted agents has been undertaken.

A human xenograft model of neuroblastoma in SCID mice was used to investigate the effect of combination

treatment of metronomic vinblastine with DC101, an anti-VEGFR2 antibody[28]. Each drug alone produced only a transient tumor inhibition. In contrast the combination resulted in sustained xenograft growth inhibition with no signs of resistance development up to seven months on treatment. In addition, some of the animals were followed after discontinuation of the combination treatment and showed no evidence of tumor recurrence. Treatment was well-tolerated and mice did not display any of the usual signs of toxicity such as weight loss, anorexia, dehydration or skin ulcerations. In agreement with the clinical effect, the combination showed more pronounced apoptotic cell death and angiogenesis inhibition than control or monotherapy treatment in histopathologic sections examination[28].

The same investigators expanded the above findings to a human orthotopic breast cancer model of cell lines MDA-MB-231 and MDA-MB-435 and multidrug resistant derivative lines expressing multidrug resistance pgp glucoprotein[29]. Continuous low dose of vinblastine, cisplatin or doxorubicin in combination with the same anti-VEGFR2 antibody resulted in improved and sustained anti-tumor effects compared with the chemotherapeutics alone while the antibody by itself had an intermediate effect.

Human colorectal cancer cell line KM12SM growing as xenograft in nude mice was investigated as a target of conventionally-dosed irinotecan or the same drug given in a metronomic schedule with or without bevacizumab[30]. Both treatments including metronomic irinotecan (with or without bevacizumab) resulted in greater anti-angiogenic effects compared with conventional schedule as measured by microvessel density and CD31 immunostaining and were better tolerated as measured by a decreased weight loss. Moreover the addition of bevacizumab appeared to act additively to metronomic irinotecan further delaying tumor growth compared with irinotecan monotherapy[30].

Another gastrointestinal tumor studied in a preclinical human xenograft model in mice is pancreatic cancer[31]. In this case the combination of metronomic schedule gemcitabine with sunitinib had specific efficacy in decreasing metastatic progression while its effect on the primary tumors was less than the effect with conventional maximal tolerated gemcitabine schedule.

Still in the realm of gastrointestinal tumor models, human hepatocellular cancer xenografts of the Hep3B cell line were studied in immunocompromised mice[32]. Treatment with sorafenib, a drug that is used clinically in the treatment of the disease, eventually led to tumor resistance and increase in tumor burden despite continuous treatment. Co-administration of a metronomic dose of UFT (15 mg/kg per day continuously) could delay the development of resistance and prolong the median survival of mice from less than 3 to 4.5 mo without increased toxicity[32]. In another study from the same group, orthotopically implanted hepatocellular carcinoma xenografts of the same cell line Hep3B were more efficiently controlled by the combination of metronomic UFT or cyclophosphamide and the anti-VEGFR2 antibody DC101 than with either agents alone[33].

In mice bearing human prostate cancer and rat gliosarcoma cell line xenografts, the VEGFR inhibitor axitinib in combination with metronomic cyclophosphamide displayed improved anti-tumor activity in comparison with either drug alone and this despite decreased tumor uptake of the active cyclophosphamide metabolite 4-hydroxy-cyclophosphamide[34,35]. Axitinib was found to possess, in addition to anti-angiogenic, direct anti-tumor cell effects inducing tumor cell apoptosis and this may relate to the fact that it is, similarly to other TKIs, an inhibitor of pathways other than VEGF, as mentioned previously[35].

Ovarian cancer was the subject of a study combining oral topotecan with pazopanib[36]. Topotecan has activity and is currently used clinically in the treatment of this type of cancer while anti-angiogenesis is also a proven beneficial modality for ovarian cancer and thus the combination is of particular clinical interest. Mice bearing human ovarian cancer xenografts in their peritoneal cavity were treated with either drug alone or their combination. Metronomic oral topotecan plus pazopanib was more effective than topotecan alone in maximal tolerated dose or pazopanib alone in reducing tumor burden and in prolonging the survival of the tumor-bearing mice[36]. Similar conclusions were reached in another study of the same combination using additional ovarian cell line xenografts[37].

In conclusion, extensive pre-clinical evidence pinpoints to the activity of metronomic chemotherapy/anti-VEGF pathway inhibitors combinations in a variety of tumor types. This together with the variety of chemotherapeutics and VEGF-targeting agents used in these combinations and producing the same synergistic effect argues for a mechanism that is independent of the specific tumor and underlying molecular lesions that it bears and is consistent with an anti-angiogenic and immune mediated mechanism.

CLINICAL STUDIES

Glioblastoma multiforme (GBM) is the most common and most aggressive malignant primary brain tumor for which the median survival with trimodality treatment followed by temozolomide maintenance is 15 mo with less than 10% of patients alive at 5 years[10]. Since several preclinical models showed up-regulation of VEGF in GBM cell lines, targeting angiogenesis has been a subject of several clinical trials that led to the approval of bevacizumab as monotherapy for recurrent GBM[38]. Few of these studies attempt to explore the role of metronomic chemotherapy in combination with angiogenic agents in this context and have attempted to bring these combinations in the forefront of GBM therapeutics.

In the front line treatment, two phase Ⅲ randomized trials showed that incorporation of bevacizumab

to the traditional radio-chemotherapy regimen with temozolomide followed by bevacizumab until progression improves the PFS, but without any benefit in OS[39]. The Radiation Therapy Oncology Group (RTOG) 0825 trial included 637 patients who were randomized to receive standard chemo-radiotherapy with temozolamide plus either placebo or bevacizumab (10 mg/kg every 2 wk)[39]. All patients had a Karnofsky Performance Scale score of at least 60. There was no statistically significant benefit of the addition of bevacizumab for the OS (15.7 mo *vs* 16.1 mo, $P = 0.21$) but a significant benefit for the PFS (10.7 mo *vs* 7.3 mo, $P = 0.007$) was noted. It is important to note that patients from the placebo group were allowed to cross over at progression, which may explain the lack of benefit in OS.

AVAglio was an industry-sponsored study conducted mainly in Europe[14]. It had a similar design that included provision to crossover on progression. It randomized 921 patients and, similarly to RTOG 0825, showed a PFS benefit for the addition of bevacizumab (10.6 mo *vs* 6.2 mo, $P < 0.001$) but failed to show an OS benefit (16.8 mo *vs* 16.7 mo, $P = 0.10$). The two trials showed contrasting results regarding quality of life (Qol) effect of bevacizumab. In the RTOG study addition of bevacizumab worsened patient neurocognitive function, while AVAglio showed improvement of Qol, prolonged time to Karnofsky Performance Scale score worsening, as well as delay in the initiation of corticosteroid treatment. One possible reason for this discrepancy between the two trials is that the AVAglio study did not incorporate a measure of neurocognitive functioning into its evaluation for Qol outcomes.

Based on the fact of failure to demonstrate benefit in terms of OS, several oncologists believe that there are not enough data to support addition of bevacizumab in front line therapy and reserve its use in large, bulky tumors with significant associated edema. Currently, Japan is the only country to approve the addition of bevacizumab in newly diagnosed GBM (http://www.roche.com/media/ media_releases/med-cor-2013-06-17.htm).

In recurrent GBM, phase Ⅱ studies have demonstrated response rates of 63% and 6 mo PFS of 38% to 46% when bevacizumab is combined with metronomic irinotecan adjusted according to whether patients were taking metabolic enzyme-inducing anticonvulsants[40,41]. In another phase Ⅱ study response rate was 47.3%, including 2 patients with complete response[42]. A phase Ⅱ trial evaluating biweekly bevacizumab with metronomic etoposide in recurrent malignant glioma[43] included 27 (of 59 total) patients with GBM. The six months PFS was 44.4%, but the toxicity was increased with the combination compared with previous reports of bevacizumab monotherapy. Interestingly low expression of VEGF in tumors assessed by immunohistochemistry was correlated with poorer PFS[43]. Another phase Ⅱ trial closed at the interim analysis as the addition of bevacizumab on metronomic chemotherapy (temozolomide or etoposide) was found to be ineffective[44].

There are limited data concerning VEGFR tyrosine kinase inhibitors in GBM. A phase Ⅱ trial has showed that the combination of sorafenib with temozolamide in recurrent GBM in 43 naïve patients for anti-angiogenic treatment is feasible and safe[45]. Twenty-three out of 43 patients had radiologically stable disease or a partial response and the median PFS was 3.2 mo and the median OS was 7.4 mo. Of note, 11% of patients suffered from a grade 3-4 hand foot syndrome.

In ovarian cancer, the activity of anti-angiogenic therapy with bevacizumab is well documented both as monotherapy (RR 21% in a phase Ⅱ study[46]) and as combination with chemotherapy. Several trials[12,47] have shown a clear benefit in PFS after incorporation of anti-angiogenesis therapy in the management of newly diagnosed advanced ovarian cancer treated with carboplatin and paclitaxel, making it a standard of care option in 1st line.

The concept of metronomic treatment with antiangiogenic therapy has been well studied in case of platinum resistant recurrent patients. A randomized phase III study comparing chemotherapy (weekly paclitaxel, weekly topotecan or liposomal doxorubicin) to chemotherapy plus bevacizumab confirmed the benefit of the combination with doubling of median PFS (6.7 mo *vs* 3.4 mo)[48]. Mature OS data presented in ESMO 2013 congress did not show statistically significant OS benefit of the combination ($P = 0.174$), despite a 3 mo survival advantage of the bevacizumab arm (13.3 mo *vs* 16.6 mo)[49]. Interestingly, in the subgroup analysis a more pronounced impact was noticed when the bevacizumab was combined with weekly paclitaxel (13.2 mo *vs* 22.4 mo), a chemotherapy regimen with anti-angiogenic activity, supporting the idea of the efficacy of the concept[49].

Moreover, a recent retrospective trial of bevacizumab and oral metronomic cyclophosphamide in heavily pretreated platinum resistant ovarian cancer, with 66 patients, has shown that the combination is active with a response rate of 42.4%[50].

The activity of weekly topotecan and biweekly bevacizumab in ovarian cancer has been shown in a trial of bevacizumab 10 mg/kg administered on days 1 and 15 and topotecan 4 mg/m^2 on days 1, 8, and 15 of a 28-d cycle until progressive disease (PD) or excessive toxicity[51]. Median PFS and OS were 7.8 and 16.6 mo respectively, with 22 of 40 (55%) of the patients being progression-free for ⩾ 6 mo. Ten (25%) patients had a partial response (PR), 14 (35%) had stable disease (SD), and 16 (40%) had PD.

A phase Ⅰ/Ⅱ study of sorafenib associated with weekly topotecan in patients with platinum-resistant ovarian cancer or primary peritoneal carcinomatosis has shown some efficacy[52]. In this study, 16 patients were enrolled in a phase Ⅰ part and 14 patients in a phase Ⅱ part. The phase Ⅱ regimen consisted of sorafenib 400 mg daily and topotecan 3.5 mg/m^2 weekly on days 1, 8, 15 of a 28 d cycle. There were 5 PR (16.7%), and 14 patients (46.7%) with SD. Nevertheless, the combination of sorafenib and topotecan caused significant toxicity.

Other studies are ongoing evaluating combinations of therapies targeting the VEGF pathway and metronomic chemotherapy in ovarian cancer. For example, a German phase Ⅰ/Ⅱ trial combines metronomic cyclophosphamide with pazopanib[53] and a phase Ⅱ study to open in United Kingdom (NCT01610869) will combine nintedanib (formerly BIBF 1120, a receptor tyrosine kinase inhibitor blocking signaling through VEGFR, PDGFR, and FGFR, also investigated in idiopathic pulmonary fibrosis[54]) and metronomic daily cyclophosphamide in patients with multiply-relapsed advanced ovarian cancer.

In breast cancer, a phase Ⅱ study of 46 patients (19 of whom had previous chemotherapy for metastatic disease) investigated the combination of metronomic cyclophosphamide at a dose of 50 mg daily with capecitabine 500 mg three times daily and bevacizumab 10 mg/kg every two weeks[55]. It showed a response rate of 48% and a clinical benefit rate of 68%. The clinical benefit was more pronounced in hormone receptors positive disease. The treatment was very well tolerated with the only grade 3 or higher adverse effect occurring in more than 10% of patients being hypertension[55]. The same investigators added erlotinib to the above combination in patients with metastatic breast cancer poorly expressing hormone receptors and negative for Her-2 and observed a response rate of 62%[56].

A phase I study with 20 metastatic breast cancer patients and up to four previous lines of treatment associated metronomic cyclophosphamide 50 mg daily with methotrexate 2.5 mg 2 d per week and vandetanib found to have a maximal tolerated dose of 200 mg daily[57]. Toxicity was acceptable and the clinical benefit rate was 25%. A similar combination with cyclophosphamide and methotrexate as the metronomic chemotherapy backbone and bevacizumab instead of vandetanib produced a clinical benefit rate of 31.8% in another study[58].

Several studies have been published showing clinical activity of metronomic schedules in advanced stage non-small cell lung cancer with acceptable toxicity profile. Data on combinations with anti-angiogenic treatments are less abundant and few studies have been published and few are ongoing. A phase Ⅱ study published only in abstract form so far presented data on the combination of two chemotherapeutics at metronomic doses (paclitaxel 80 mg/m^2 weekly three weeks out of four and gemcitabine 200-300 mg/m^2 also weekly three weeks out of four) with bevacizumab (10 mg/kg biweekly). Maintenance bevacizumab was an option for patients with a good tolerance and no progression. The trial showed a median OS of 30 mo and a 2-year OS of 55% in advanced non-squamous lung cancer[59].

A small pilot phase Ⅱ study combined a metronomic oral chemotherapy part of etoposide at 50 mg per day for 14 d of a 21 d cycle and bevacizumab at 5 mg/kg with a more intense part of cisplatin at a dose of 30 mg/m^2 for 3 consecutive days. This was followed by maintenance of erlotinib and bevacizumab in case of stable disease or response[60]. The combination demonstrated a 69% partial response rate and 86% disease control rate (partial response or stable disease). The PFS was 9.53 mo. Toxicity was also significant as expected from a regimen with a more intense component and included grade 3 or 4 myelotoxicity in 15% of patients and grade 3 or 4 GI toxicity in 18%[60].

A phase I study of the combination of metronomic vinorelbine at a starting dose of 40 mg three times per week with sorafenib (NCT00870532) has been completed but results have not been published yet.

Clinical data from studies in other tumor types, although less abundant, also confirm the concept of the combination. A study of patients with hepatocellular carcinoma and Child-Pugh class A liver function combined sorafenib with metronomic tegafur-uracil and showed a clinical benefit rate of 57%[61]. In a study of metastatic colorectal cancer patients who had at least two previous lines of therapy, the combination of cyclophosphamide 50 mg daily with imatinib 400 mg daily and bevacizumab 5 mg/kg every 2 wk was well tolerated and led to prolonged (more than 6 mo) stabilization of the disease in 20% of patients[62]. In a small study that included 15 patients with malignant neuroendocrine tumors mainly of gastrointestinal origin, the combination of temozolomide 100 mg daily with a long acting somatostatin analogue and bevacizumab produced a 64% response rate and 86% clinical benefit rate[63].

THE WAY AHEAD: PREDICTION OF RESPONSE

As witnessed by other cancer treatments such as anti-Her2 antibodies, the presence of a robust biomarker of response greatly facilitates the development and establishment of a drug in the clinic. The absence of such confirmed biomarkers for anti-VEGF treatments and for metronomic chemotherapy hampers their development in the clinic and has certainly contributed to negative results of trials in some type of cancers and moderating their success in others. Extensive investigations on discovering such biomarkers have not succeeded in bringing any biomarker forward to clinical applicability so far. Initial exploratory investigation on the predictive value of VEGF single nucleotide polymorphisms identified two minor alleles associated with bevacizumab response in a breast cancer population but this was not consistently seen in another study also in breast cancer[64]. Circulating stem or endothelial progenitor cells and MRI imaging parameters have been proposed as markers of response to sorafenib and bevacizumab[65,66]. The previously discussed report on metronomic cyclophosphamide, capecitabine and bevacizumab in breast cancer found a higher response of this treatment in patients with higher baseline circulating endothelial cells[55]. K-ras mutations, which predict for lack of response to EGFR targeting therapies in metastatic colorectal cancer, are not predictive of response to bevacizumab despite k-ras being also part of one of the intracellular signal cascades triggered by VEGFR[67]. This

argues for the importance of indirect mechanisms in the cytotoxicity of VEGF treatments. Prediction of which patient will respond to a given metronomic chemotherapy treatment is similarly currently unfeasible.

Another interesting biomarker that has been proposed as a predictor of response to bevacizumab is thrombocytosis[68]. This is a well validated laboratory value already routinely used in the clinic and has been associated with adverse outcomes in a variety of tumor types[69-72]. Interleukin-6 (IL-6), a thrombopoiesis-promoting cytokine, production by the tumor is implicated in the induction of thrombocytosis[70]. IL-6 is concomitantly an angiogenesis-promoting cytokine and thus its presence may denote a particular angiogenic propensity of tumors and dependence, as a result, to angiogenic pathways. In addition, platelets contribute to this effect by carrying additional pro-angiogenic substances in their granules[73]. As a result their number in a particular patient may represent a marker of the tumor dependency to a combination of treatments such as anti-VEGF and metronomic chemotherapy that rely on inhibition of angiogenesis as a mechanism of their action. Platelet proteome has been reported to change after treatment with metronomic cyclophosphamide, methotrexate and vandetanib[58] but it is unknown if the baseline status of this proteome predict for response to treatment. Platelet number and content represent interesting predictive markers for further investigation for the combination. Confirmation of their value and discovery of other useful predictive biomarkers will certainly facilitate a wider adoption of combinations of VEGF therapies and metronomic chemotherapy that could be a valuable option especially in later line of therapy where lower toxicity therapies are needed to fit with the profile of patients with lower performance statuses.

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