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Perspective

The DNA Damage Signaling Pathway Connects Oncogenic Stress to Cellular Senescence

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ABSTRACT

The mechanisms of tumor suppression must be linked to the oncogenic threats that may affect a normal cell. An important cancer causing mechanism is the accidental activation of genes that stimulate cell proliferation (oncogenes) by a variety of endogenous or environmental mutagens. This event has been experimentally modelled by enforcing the expression of oncogenes in primary cells. The astonishing outcome of these manipulations is that oncogenes trigger antiproliferative responses preventing progression to malignant transformation. These responses bring to an end proliferation due to cell death or a permanent cell cycle arrest called senescence. Here we review evidence indicating that oncogene induced senescence (OIS) involves activation of p53 via the DNA damage response (DDR). These results imply mechanisms of DNA damage in cells expressing oncogenes, that may be secondary to reactive oxygen species and/or some form of "oncogenic stress" that affect normal DNA replication. Interestingly, DNA damage signals persist in cells that escape from senescence. The implications of these signals for tumorigenesis are also discussed. Given that DNA damage signals have now been observed in cells treated with any stimuli known to induce senescence, the process can be redefined as a metabolically viable but permanent cell cycle arrest with persistent DNA damage signaling.

INTRODUCTION: ONCOGENES AND TUMOR SUPPRESSORS

Cancer is a common disease in human and other mammals. However, most individuals succumb to cancer late in their life span.[1] The study of cancer cells revealed that they contain multiple mutations and chromosomal aberrations.[2,3] It has been rationalized that a long period of time is required for any individual cells to accumulate the right combination of mutations that promote the cancer cell phenotype. Mutations consistently found in cancer cells are selected because they confer a growth advantage by either activating growth promoting pathways, inactivating growth inhibitory pathways or allowing mutations to accumulate.[4] Genes found mutated in human cancers promoting cell growth are known as oncogenes.[5] Oncogenes are altered version of normal genes (proto-oncogenes) and their activation is accidental.[4,5] Therefore, over the life span any individual may acquire multiple mutations with oncogenic potential, yet only a fraction of individuals experience cancer late in their life span. This fact of life suggests that organisms evolved mechanisms to prevent oncogenic transformation. These mechanisms are controlled by the so called antioncogenes or tumor suppressor genes.[6]

Tumor suppressors may avert cancer by preventing mutations (stability genes), inducing cell death or a program of cell division arrest known as cellular senescence.[4,7] A key question is: How tumor suppressor proteins recognize an oncogenic threat in the cells? Recent studies, using a variety of oncogenes that induce cellular senescence, identified a common pathway connecting oncogenes to tumor suppressors and the senescence program.

ACTIVATION OF p53 BY ONCOGENES: ROLE OF DNA DAMAGE

The activation of p53 by oncogenes was first observed in studies of oncogene-induced apoptosis and later confirmed in oncogene-induced senescence (OIS).[7] However, the mechanistic details of the connections remained unknown. The discovery of p19ARF as a p53 activator required for p53 induction by several oncogenes suggested that ARF acted as a general oncogenic sensor.[8-10] However, ARF is not in reality a sensor in itself because oncogenes regulate ARF at the transcriptional level. It remains to be investigated how

the proteins that regulate ARF expression[11] recognize an oncogenic stress. Intriguingly, the ARF locus is located between the tumor suppressors p15INK4b and p16INK4a in human chromosome 9, sharing some sequences with the p16INK4a gene. The three loci are regulated by a replication origin placed 1.5 kb upstream of the start codon of p15INK4b. The replication protein Cdc6 binds this origin and promotes the repression of the INK4/ARF locus.[12] It would be interesting to examine whether expression of the INK4/ARF locus in response to oncogenes involves a suppression of this Cdc6-dependent repression mechanism.

It has been puzzling to recognize that ARF is not required for OIS in human cells.[13] Studies from our laboratory and the laboratories of Gorgoulis and D'Adda di Fagagna demonstrated that the DNA damage signaling response (DDR) was required for the activation of p53 in response to a variety of oncogenes, including RasV12, STAT5, E2F1, Mos and Cdc6.[14-16] These papers were in accord in demonstrating that the DDR kinases ATM, ATR, Chk1 and Chk2 were implicated in OIS and their inactivation by RNA interference contributed to bypass OIS. Collectively, these papers argue that oncogenic activity leads to DNA damage, providing a signal to organize an antioncogenic response. Consequently, proteins that acts as oncogenic sensors, could be detecting DNA damage or its immediate effects. Interestingly, the ability of ARF to induce p53 in human osteosarcoma cells was also dependent on the DDR kinases ATR and Chk1.[17] It may well be that both ARF and the DDR are part of an interconnected cellular network to regulate the cellular response to oncogenic threats.

DNA DAMAGE FOCI IN SENESCENT CELLS

The revealed links between oncogenes and the DDR in normal cells suggest that some form of DNA damage is induced by oncogenes. Several forms of DNA damage can be easily detected in cells as foci that accumulate proteins of the DDR and histone modifications such as phosphorylation of the histone variant H2AX.[18] These foci were observed in both primary cells expressing oncogenes[14-16] and also in early pre-malignant lesions.[19-21] We named the DNA damage foci found in senescent cells ODDI (oncogene-induced DNA damage foci). We think these foci may be unique because they are persistent. Unlike DNA damage foci seen in cells treated with radiation or drugs, ODDI could be resistant to repair. In addition, ODDI are not associated to telomeres[16] and are therefore different from DNA damage foci reported during replicative senescence.[22] In agreement, OIS does not to depend on short telomeres because expression of telomerase can not inhibit the process.[14,15,23] What is then the cause of formation of ODDI and activation of DDR in oncogene-expressing cells?

DNA damage foci and DNA replication stress. One intriguing observation is that forcing oncogene-expressing cells to arrest in G_1, by serum starvation, prevented the induction of the DDR by Cdc6.[15] Similarly, inhibition of DNA replication with aphidicolin in cells expressing oncogenic *ras* prevented the activation of the DDR.[14] These results could suggest that some sort of replication stress is required to induce the DDR. The DNA damage foci induced by Cdc6 or cyclin E in U2OS ostesarcoma cells or BJ normal fibroblasts co-localized with RPA, a protein that binds single stranded DNA (ssDNA) during DNA replication. However, RPA can also bind to ssDNA during DNA repair in G_1.[24] A more direct support for the replication stress model was provided using a technique able to detect replication forks that prematurely arrest or pause their progression.[14,15] Arrested forks are known to collapse giving rise to double strand breaks that appear as DNA damage foci.[25]

Another kind of replication stress able to induce the DDR is DNA re-replication. This kind of replication stress was detected in Ras-induced senescence using fluorescence in situ hybridization. It was found that OIS cells often contained more than two copies of two chosen loci, indicating re-replication.[14] Previous work showed that replication initiation proteins Cdc6 and Cdt1 along with Cdk2-cyclin A could induce re-replication in cancer cells but not in normal cells with intact p53.[26] It is not clear why p53 was not able to prevent re-replication during OIS in spite that p53 is clearly active during this process. Loss of Geminin was reported to favour re-replication despite a normal p53 activity.[27] Hence, it would be interesting to investigate whether Geminin functions are compromised during OIS.

One unresolved issue with the replication stress model is that the immediate consequence of a re-replication stress should be an S phase arrest, the triggering of the G_2 checkpoint or death in mitosis.[27,28] However, there is no significant cell death during OIS and according to DNA content analysis senescent cells are mainly arrested in G_1.[29,30] Currently, there are two plausible explanations for this question. The first possibility is that cells carry replication stress-induced damage through S, G_2 and M to arrest in G_1. The other possibility is that DNA content analysis is not sensitive enough to detect cells at the G_1/S transition when some replication origins already fired.

ODDI AND TRANSCRIPTION

All oncogenic activities eventually lead to an increase in transcription of specific genes. Transcription could lead to DNA damage in different ways. Studies in yeast suggest that DNA breaks induced by the transcription machinery can increase recombination.[31] In support of this idea it has been shown that DNA topoisomerase IIb is associated to promoters in mammalian cells where it can generates DNA breaks.[32] These topoisomerase-mediated breaks activate the enzyme poly-ADP ribose polymerase, which induces a replacement of histone H1 by the high mobility group protein (HMG) B1/2. Intriguingly, during Ras-induced senescence the histone H1 is replaced HMGA2 suggesting a mechanism by which DNA breaks can lead to chromatin changes during senescence.[33]

ODDI AND REACTIVE OXYGEN SPECIES

The data supporting a replication stress model do not preclude that other kinds of DNA damaging stresses contribute to OIS. Senescent cells have higher levels of reactive oxygen species than normal cells growing in the same conditions.[34] In addition growing normal human cells in 1% oxygen prevented Ras-induced senescence and p53 activation.[34] Cells that undergo senescence due to short telomeres also display high levels of ROS.[34,35] In fact, telomerase can not immortalize all isolates of human diploid fibroblasts when cells are grown in 20% oxygen.[36] These data indicate that replicative senescence is not only driven by the shortening of telomeres, but also by DNA damage induced by ROS. In fact, ROS can directly damage telomeres accelerating their erosion.[37] Taken together, these data suggest that ROS may also contribute to both OIS and replicative senescence.

ROS can be produced by mitochondrial electron transport. However, in Ras-induced senescence, ROS derived from the action of 5-lypoxygenase seem to also play a role.[38] It is commonly assumed that ROS may engage the p53 tumor suppressor pathway

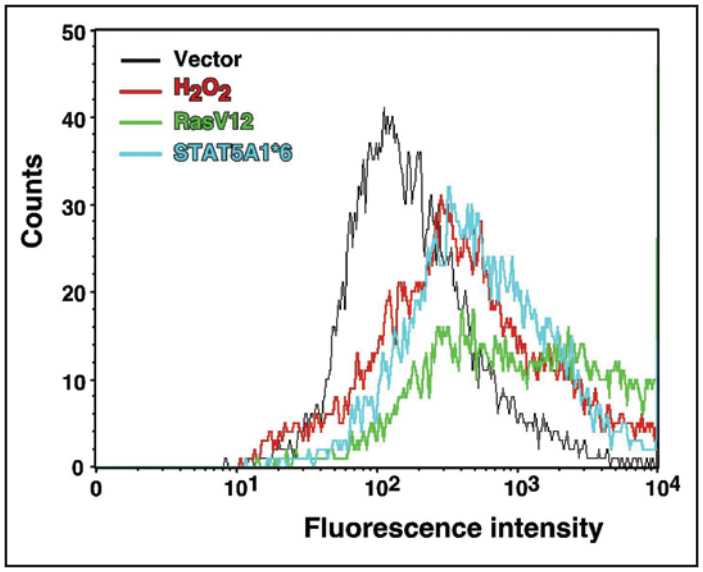

Figure 1. Accumulation of ROS in cells expressing two different oncogenes that induce senescence: RasV12 and ca-STAT5A. The levels of ROS are similar to those found in cells treated with a 100 μM H2O2. Senescence was induced as (described in ref. 16) and ROS were measured by FACS using the fluorescence probe H2DCFDA from Molecular Probes as (described in ref. 73).

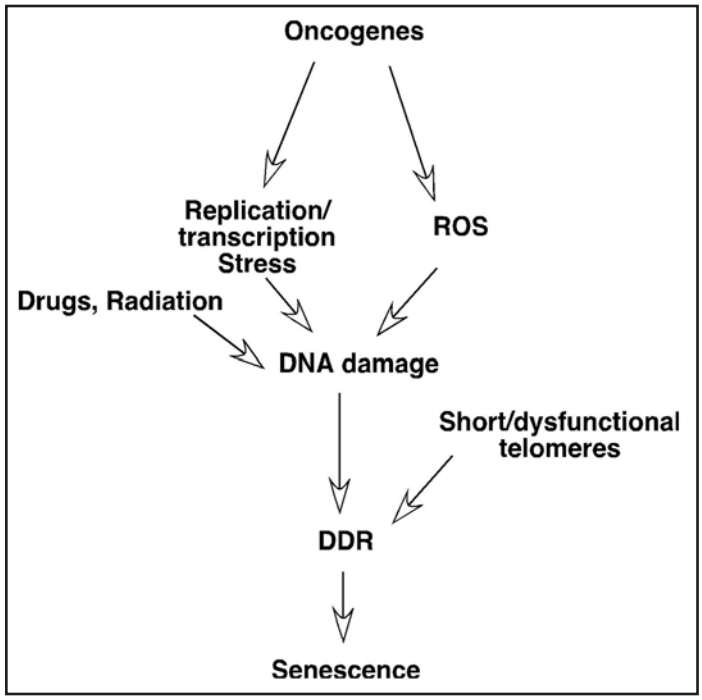

Figure 2. Senescence a general response to DNA damage signals: oncogenes may induce DNA damage by forcing an aberrant DNA replication process where some replication forks arrest as a result of some yet unknown mechanisms. Another possibility is that oncogenes induce the accumulation of ROS, which are known for their reactivity and ability to produce oxidative base damage and DNA breaks.

by inducing DNA damage. ROS-induced DNA damage includes both single stranded and double stranded DNA breaks. In addition, ROS directly induce oxidative DNA damage and this kind of damage is highly increased in senescent cells.[39] These data indicate that ROS-induced DNA damage may contribute to the senescence phenotype. In support of this model, treatment of primary cells with hydrogen peroxyde induces DNA damage, p53 activity and senescence.[40] However, the relationship between ROS, DNA damage and p53 is more complex. The Myc oncogene can induce ROS accumulation and DNA damage signals. However, at the same time Myc blocks p53 activity permitting DNA replication with DNA damage, which may induce genomic instability.[41] We have detected high levels of ROS in cells expressing oncogenic Ras or constitutively active STAT5A (Fig. 1). These oncogenes affect different signaling pathways, suggesting that the ability to induce ROS is a general trait of oncogenic activity.

ROS may play a more complex role in senescence due to their ability to modify protein activity. For example, ROS can activate Seladin, a protein that binds the N-terminus of p53 interfering with binding of Mdm2 and preventing the degradation of p53.[42] ROS can also activate protein kinase C δ (PKCδ), which in turn increases ROS production by activating NADPH oxidase, a ROS generating enzyme. These interactions form a positive feedback loop controlled by p16INK4a. Downregulation of p16INK4a using RNAi blocked senescence, ROS production and activation of PKCδ in cells with short telomeres.[43] This mechanism seems to be relevant for OIS, because high levels of ROS and PKCδ were also detected in cells expressing oncogenic *ras*.[43] In addition PKCδ phosphorylates WARTS leading to its degradation. WARTS is a protein kinase required for cytokinesis. In agreement with a role for PKCδ in OIS, WARTS levels are low in OIS and OIS is associated with a significant increase in polyploid cells.

ROS levels in cells are a consequence of a balance between ROS production and detoxification mechanisms. p16INK4a increased ROS levels, at least in part, via downregulation of E2F-dependent expression of ROS detoxifying enzymes such as MnSOD.[43] In senescence induced by a constitutive allele of AKT, inhibition of FOXO3a by AKT resulted in accumulation of ROS.[44] FOXO3a is another well-known regulator of MnSOD expression.[44] These data indicates that reducing the normal defence against ROS can have a causal role in senescence. In agreement, direct ablation of SOD by RNA interference induced cellular senescence.[45] Together, the available data support a model where both replication stress and ROS contribute to OIS perhaps converging on the DNA (Fig. 2).

p53, RB AND THE DNA DAMAGE RESPONSE (DDR)

The role of the DDR in OIS was different in the studies of Bartkova et al. and DiMicco et al., in comparison with the work coming form our laboratory. While Bartkova et al. or DiMicco et al. could bypass OIS by interfering the DDR, we only observed a rescue of OIS by inactivation of the DDR in the context of inactivation of the Rb tumor suppressor pathway. Earlier work on OIS established that in human cells the Rb and the p53 pathways had to be simultaneously inactivated to bypass OIS.[29,30] This original work was done in the strain of normal fibroblasts IMR90, which are derived from embryonic lungs. In contrast, in BJ foreskin fibroblasts, it has been reported that inactivation of p53 is sufficient to bypass OIS.[46] In agreement, inactivation of the DDR in these cells also bypassed OIS.[14,15] One explanation to reconcile these results is that the Rb pathway is not as active in BJ foreskin fibroblasts as in IMR90 cells. Since the p16/Rb pathway controls ROS production[43] it would be interesting if differences in the ability of oncogenes to induce ROS

may explain the different results obtained between different isolates of human diploid fibroblasts. In fact, certain isolates of normal human diploid fibroblasts are resistant to Ras-induced senescence and have very low levels of p16INK4a.[47] Again, it would be interesting to study whether oncogenes can induce ROS or the DDR in these cells.

Another characteristic of cellular senescence that has been less well studied is the stability of the phenotype. It has been proposed that senescent cells are stably arrested because genes required for cell proliferation are locked in heterochromatin structures known as SAHF (senescence associated heterochromatin foci).[48] Intriguingly, SAHF, defined as DAPI-stained nuclear foci, are not present in every form of senescent arrest. BJ fibroblasts exhibit little evidence of SAHF formation in response to oncogenic *ras* and lower expression of p16INK4a in comparison with the embryonic lung fibroblasts IMR90. One idea is that SAHF and p16 control the reversibility of senescence. In agreement, inhibition of p53 could reverse senescence in BJ cells but not in IMR90 cells.[49] The relationship between the DDR, p16INK4a and SAHF remain unexplored, but it is certainly possible that they are all linked.[50]

SENESCENCE: A METABOLICALLY VIABLE STATE OF PERSISTENT DDR

The senescent phenotype was originally observed after serial passage of primary cells in culture.[51] Repeated cell division with cell passage leads to telomere erosion, because human somatic cells do not express the enzyme that replenishes telomeres.[52] Short telomeres trigger a local DDR, suggesting that they must look like double strand breaks.[22,53] Since oncogenes also induce DNA damage signals[14-16] and DNA damaging agents can directly induce senescence,[39,54] it is applicable to define senescence as a metabolically viable cell cycle arrest with persistent DNA damage signaling.

Senescence cells are known to be metabolically active and are able to secrete a variety of inflammatory mediators.[55] The constitutive activation of the DDR observed in these cells raises questions about how the DDR affect transcriptional programs involved in the stress response, cell differentiation or the response to environmental signals. Cells with constitutive activation of the DDR have been detected in old organisms.[56,57] It has been proposed that these cells may contribute to aging via its secretion products.[58,59] OIS provides a convenient cell culture model to study how a persistent DNA damage signal modifies the proteome and transcriptional programs with the potential to interfere with both cell functions and tissue homeostasis.

ESCAPE FROM SENESCENCE AND DDR

The presence of DNA damage foci characterizes both advanced human cancers and pre-malignant lesions.[19-21] Since those tumors must have already found a way around the senescent barrier, we reasoned that bypassing oncogene-induced senescence might not eliminate ODDI. We found that cells that evade senescence, due to expression of viral oncoproteins, proliferate freely in the presence of ODDI.[16] There are three important implications from this result.

First, comes the question of how do cells manage to proliferate in the presence of DNA damage signals. One idea comes from the concept of adaptation proposed by Sandell and Zakian to explain why yeast cells could grow in the presence of DNA damage signals generated by the loss of telomeres.[60] During this process the activity of the kinases Chk1 and Chk2 (Rad53) is actively suppressed.[61] The mechanism depends on yKu70, casein kinase II (CSKB) and the polo like kinase (Cdc5).[61,62] The role of the polo like kinase in adaptation has been confirmed in Xenopus, indicating that this pathway is conserved from yeast to vertebrates.[63] In mammals, B cells undergo a physiological double strand break that could activate a checkpoint response. In this condition, cells avoid the response via a Bcl6 dependent repression of p53 expression.[64] Intriguingly, Bcl6 has oncogenic activities and is able to bypass Ras-induced senescence.[65] Similar to Bcl6, the Kruppel-like factor 4, KLF4, represses p53 transcription and inhibits Ras-induced senescence.[66] Together, these studies suggest that multiple molecular mechanisms may contribute to suppress DNA damage signals in tumor cells.

The second implication is that ODDI may still contribute to tumor suppression in cells that evade senescence. In fact, it is known that bypassing senescence is not sufficient to transform primary cells.[67-69] Some results suggest that the G_2 checkpoint may offer tumor protection in cells where the G_1 checkpoint is inhibited. For example, cells expressing Myc do not arrest in G_1, despite having DNA damage signals,[41] however, they do arrest in G_2.[70] In addition, a p53-dependent G_2 arrest prevented tumorigenesis in cells disabled for the Rb family, which are unable to arrest in G_1.[71]

The third implication is that oncogene-induced DNA damage may actually stimulate malignant progression once the senescence barrier and other negative regulators of cell proliferation are disabled. Studies with primary mammary epithelial cells indicated that these cells, in contrast to fibroblasts, can readily escape from senescence accumulating multiple genomic changes that may accelerate tumor progression.[72] Fibroblasts may be more resistant to transformation than epithelial cells, explaining why most cancers affect the latter cell type.

Clearly, the effects of oncogenes, ROS and DNA damage are context dependent. In normal cells they trigger antiproliferative responses but in cells where those responses have been inhibited by genetic or epigenetic mechanisms, they actually accelerate tumor progression and stimulate aberrant cell proliferation. A practical consequence of this concept is that strategies to prevent cancer, by for example increasing DNA damage via ROS, may paradoxically stimulate incipient tumors. On the other hand, blocking ROS production to treat established tumors may help to bypass the senescence response that prevents the early stages of tumorigenesis. Cancer prevention and therapy should evolve to the design of smart devices with the ability of selective targeting.

CONCLUSIONS

The recognition of DNA damage and DNA damage signals at the very early stages of transformation opens new experimental avenues in cancer research. Cells with a signature of DNA damage may be targeted for cancer prevention. There are also new conceptual implications. Protein involved in DNA damage signaling such as ATM, ATR, Chk1 and Chk2, were considered tumor suppressors because they avoided the accumulation of mutations (caretakers). However, since these proteins are required for the senescence program they also act preventing the expansion of cells bearing oncogenic mutations (gatekeepers). Finally, the presence of DNA damage signals associated in senescent cells and the requirement for the DDR to establish the senescent phenotype justifies to redefine senescence as a special case of cell cycle arrest with chronic DNA damage signaling.

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