Stress induced premature senescence: a new culprit in ovarian tumorigenesis?

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Received January 22, 2013

Stress induced premature senescence (SIPS) is a relative extension to the concept of exogenous cellular insult. Besides persistent double strand (ds) DNA breaks and increased β-galactosidase activity, biological significance of telomeric attrition in conjunction with senescence associated secretory phenotype (SASP) has been highlighted in SIPS. To gain insight on the potential role of this unique phenomenon invoked upon environmental stress, we sequentially validated the molecular repercussions of this event in ovarian epithelial cells after exposure to methyl isocyanate, an elegant regulator of cellular biotransformation. Persistent accumulation of DNA damage response factors phospho-ATM/γ-H2AX, morphological changes with increased cell size and early yet incremental β-gal staining, imply the inception of premature senescence. Advent of SASP is attributed by prolonged secretion of pro-inflammatory cytokines along with untimely but significant G1/S cell cycle arrest. Telomeric dysfunction associated with premature senescence is indicative of early loss of TRF2 (telomeric repeat binding factor 2) protein and resultant multiple translocations. Induction of senescence-associated heterochromatic foci formation showcases the chromatin alterations in form of trimethylated H3K9me3 in conjunction with H4 hypoacetylation and altered miRNA expression. Anchorage-independent neoplastic growth observed in treated cells reaffirms the oncogenic transformation following the exposure. Collectively, we infer the possible role of SIPS, as a central phenomenon, to perturbed genomic integrity in ovarian surface epithelium, orchestrated through SASP and chromatin level alterations, a hitherto unknown molecular paradigm. Although translational utility of SIPS as a biomarker for estimating ovarian cancer risk seems evident, further investigations will be imperative to provide a tangible way for its precise validation in clinical settings.

Key words Biomarker - environmental health - occupational medicine - ovarian cancer - translational oncology

Introduction

Cellular response to endogenous and exogenous stress is intricately regulated by a complex network of molecular signaling pathways. Environmental stress hastens the shriveling of the tips of telomeres, alters the phenotypic characteristics, bundles of genes inside, protein and metabolic processing that shortens cellular life span and accelerates the process of premature senescence. While cellular senescence is primarily believed to be a protective mechanism against cancer, it has also been hypothesized that the accumulation of premature senescent cells in tissue microenvironment
may contribute to persistent double strand DNA breaks, mitochondrial oxygen radical injury, sustained clinical grade inflammation and abnormal proliferation\(^1\). Replicative senescence is discovered as a process that can limit the life span of cells by reducing their proliferation\(^2\). Significant progress has been made in deciphering the major regulatory pathways that control this physiologic programme. Cells undergoing stress induced premature senescence (SIPS) share many cellular and molecular features as those undergoing replicative senescence, but they differ in the aspect of the time at which these features exhibit. While replicative senescence is regulated by biological clock that relates to progressive shortening of repetitive DNA sequences (TTAGGG) called telomeres that cap the ends of each chromosomes, the latter is not programmed but is a biological manifestation of senescent features imposed upon stress\(^3\). The role of SIPS is now gaining wide prominence due to the fact that it is a relative extension to the concept of exogenous insult to a cell. To gain insight on the potential role of this unique phenomenon invoked upon environmental stress, we sequentially delineated the molecular repercussions of this event in ovarian epithelial cells using methyl isocyanate (MIC), an established agent of oxidative radical injury. We also aimed to provide a direct role of SIPS mediated genetic and epigenetic alterations, as central phenomenon, to perturbed genomic integrity in ovarian surface epithelium, a hitherto unknown molecular paradigm.

Environmental stressors such as isocyanates, a group of low molecular weight aromatic and aliphatic compounds containing the functional isocyanate group (-NCO), are important raw materials with diverse industrial applications. A reactive industrial by-product, methyl isocyanate (MIC) is one of the most toxic isocyanates and is known to exert a detrimental effect on numerous organ systems and cellular organelles\(^4\). Due to nature and primary property, it binds to human tissues, proteins and DNA, forming toxic adducts and metabolites\(^5\). Through a series of systematic experiments conducted over the last decade, we have demonstrated that MIC is capable of eliciting, double strand DNA breaks\(^6\), mitochondrial oxidative stress\(^7\), pro-inflammatory cytokine response\(^8\), chromosomal and microsatellite instabilities\(^9\)-\(^12\). These comprehensive mechanistic investigations coupled with molecular epidemiological studies conducted in defined population cohorts exposed to MIC during the infamous Bhopal gas tragedy have further expounded both dose-exposure and exposure-response relationship at the level of genome and the epigenome\(^13\)-\(^17\). The importance of such studies has been globally realized\(^18\)-\(^21\). MIC is now believed to orchestrate a range of respiratory, reproductive, neurologic, ophthalmic and cardiovascular human ailments including immune-dysfunction, metabolic disorders, carcinogenesis and accelerated ageing or premature senescence\(^4\).

Ovarian surface epithelium is a modified mesothelium covering the surface of the ovary separated from underlying ovarian stromal tissue by a basal lamina of collagenous connective tissue. Ovarian surface cells are consequently exposed, within a limited diffusion radius, to inflammatory agents and reactive oxidants generated during peri-ovulatory processes. Majority (95%) of ovarian cancers originate in the epithelial cells on the ovary\(^24\). A genetically altered progenitor cell, with unrepaired DNA, but not committed to death, can give rise to a transformed phenotype that is propagated. Substantial focus on the impact of environment on reproductive health during the past decade has underscored the toxic chemical exposure to the defective ovarian epithelium with reduced fertility\(^25\). This review enlists the impact of SIPS both at the level of genome and epigenome and elucidates its plausible role as an ardent player in ovarian carcinogenesis.

**SIPS in oxidative damage**

Stress induced oxidant imbalance has vital connotations for both physiological (regulating the ovarian follicular cycle) and non physiological (premature ovarian follicular atresia) effects attributing to pathological processes in the female reproductive tract\(^25\). In this regard, the presence of altered nucleoside 8-hydroxy-2-deoxyguanosine (8-OH-dG), as an oxidative DNA mutation marker, might serve as a sentinel event demonstrating oxygen radicals mediated senescence and limiting of cell growth in ovarian surface epithelium (OSE)\(^26\). Isocyanates such as MIC, though industrially indispensable, have been proven to show adverse health effects by inducing DNA damage adducts\(^6\). Besides, MIC has also been shown to cause reproductive health effects including genomic alterations of spermatogonial stem cells and OSE cells\(^4\),\(^5\),\(^10\),\(^11\). Fig. 1 describes the susceptibility of mouse ovarian epithelial cells to oxidative damage induced by MIC with accumulated 8-OH-dG in treated cells. This suggests that oxidative damage might be one of the earliest alterations in ovarian epithelial cells.
Persistent DNA damage response

Several senescence-inducing stimuli engender a sustained DNA damage response (DDR), associated with DNA double-strand breaks (DSBs)\(^9,27\). The senescent phenotype gradually develops over time after the initial DNA damage and is maintained by an ongoing DDR. Sensor proteins such as the phosphoinositide 3-kinase-like kinases (PIKKs) ataxia telangiectasia mutated (ATM) and ataxia telangiectasia Rad 3 related (ATR) initiate the DDR signaling at DSBs as focal aggregates termed DNA damage foci and the MRN (MRE11-RAD50-NBS1) complex amplify the signaling and activate further PIKKs for participation in DNA repair\(^{28}\). DNA damage foci are transient, when DNA lesions are repairable. However, in the event of severe or irreparable DNA damage, such as complex breaks or uncapped telomeres, leads to inception of senescence in many cells with persistent DNA damage foci and constitutive DDR signaling\(^1\). Deregulated DDR inclines towards segmental progeroid syndromes in mammals and the integrity of DNA of surface epithelial cells of ovary is seemingly sensitive to genotoxic insult due to its direct circumjacent to the ovarian rupture site during the ovulatory process\(^{29}\). This phenomenon has been demonstrated in MIC treated mouse ovarian epithelial cells (B/CMA.0v) through increased nuclear retention of phospho-ATM/γ-H2AX foci in cells even after 48 h of exposure (Fig. 1).\(^{10}\)

Cell cycle regulatory inhibitors

These persistent changes precede advent of senescence-associated phenotypes, including growth arrest. Excessive accrual of DNA damage responsive features causes early cell cycle arrest that includes activation of cell cycle arrest checkpoints at the G1/S phases of cell cycle\(^6,7\). Checkpoint kinase1 (CHK1) and CHK2 downstream DDR effector proteins, which initiate cell cycle checkpoints and phosphorylation of p53, predominantly maintain persistent cell cycle arrest and thus function in non-redundant manner for stress induced senescence growth arrest\(^{28}\). Active p53 is required for both emergence and maintenance of the senescence growth arrest suggesting that there is a reservoir of active p53 in senescent cells\(^{30}\). p53 activation occurs through stabilization and post-translational modifications through direct/indirect phosphorylation at N-terminus by activated ATM. As a result, transactivation of downstream factors viz. p21 and p16 occurs. p21\(^{(Waf1/Cip1/Sdi1)}\) is a well known cyclin dependent kinase (CDK) inhibitor that blocks the activity of cyclinE-CDK2 and cyclin-CDK4/6 complexes critical for progression from G1- to S-phase of cell cycle\(^{12}\). Fig. 2a depicts the sustained G1 arrest of B/CMA.0v cells due to MIC exposure. The increase in abundance of p21 happens early in senescence, while increases in p16 develop rather later. p16 inhibits cyclin D1-CDK4/6 complex required for progression from G1- to S-phase and responsible for pRb phosphorylation. In its hypophosphorylated form, pRb sequesters elongation 2 promoter binding factor (E2F) transcription factors that are required for initiation of S-phase. Stress sensor protein Gadd45 (growth arrest DNA damage 45) expression is mediated by a complex interplay of physical interactions with other cellular proteins including p53, proliferating cell nuclear antigen (PCNA), p21, cdc2/cyclinB1, and p38 and C-jun N-terminal kinases (JNK) stress response kinases and has been correlated with the appearance of senescence in response to oncogenic stress, DNA damage-induced S-phase arrest and crosslink repair\(^{31}\).
Moreover, increased intraepithelial abnormalities in ovarian milieu have been ascribed to abnormal cell cycle regulation through altered p53 protein expression. Accumulation of irreparable damage in ovarian microenvironment has been related to one of the contributory factors of premature ovarian failure. Also, Cyclin D1, which is quite uncommon in normal ovary has been shown to express aberrantly in altered ovary epithelium.

**Inception of premature senescent phenotype**

Senescent cells show distinct features to that of proliferative/pre-senescent cells in multiple aspects of cellular physiology and features of gene expression. A typical flattened enlarged cell size, characteristic of senescence, with large nucleus, increased cytoplasmic granularity and focal enrichment of lysosome-related β-galactosidase activity at vacuoles is observed. Senescent cells exhibit a different repertoire of gene expression and some of these have been used as biomarkers associated with this phenotype. A senescence associated β-galactosidase (SA-β-gal), a biomarker, is detected by histochemical staining of cells using the artificial substrate X-gal. The presence of the SA-β-gal biomarker is independent of DNA synthesis and evidently distinguishes senescent cells from quiescent...
cells. Also upregulation of cell cycle inhibitors indirectly influences cell enlargement and activation of the SA-β-gal activity. Induction of a senescence-like phenotype also manifests due to environmental and genotoxic stress stimuli and accelerates senescence prematurely in young proliferative cells. Normal ovarian surface epithelial cells are of an uncommitted phenotype. The interaction between different epithelial components of ovary milieu after exposure to environmental toxicants, initiate myriad of phenotypic changes. Among these changes increased nuclear size and, nuclear contour irregularity, nuclear-cytoplasmic ratio, presence and size of nucleoli are important. Fig. 2b depicts the emergence of premature senescence in treated B/CMA.Ov cells from the 48 h post treatment itself as demonstrated by elevated β-galactosidase stain positive cells due to an increase in lysosomal mass with altered cell morphology characterized by a giant cell size (Fig. 2c).

**Senescence associated epigenetic alterations**

Drastic chromatin changes occur during the establishment of senescence, most striking feature of which is the formation of senescence associated heterochromatin foci (SAHF) primarily described by Narita et al. SAHF are DNase resistant 4,6-diamidino 2-phenylindole (DAPI) dense, sub-nuclear cytological structures that are enriched for facultative heterochromatin. Whereas normal cells show relatively uniform DAPI staining of nuclei, senescent cells show up to 30-50 punctate DAPI-stained DNA foci. Each focus apparently represents condensed chromatin of one chromosome. SAHF also feature enriched protein modifications typical of transcriptionally silent heterochromatin, such as heterochromatin proteins 1 and histone modifications associated with lysine 9-trimethylated histone H3 (H3K9Me3). A number of additional proteins are known to contribute to formation and/or maintenance of the SAHF, including high-mobility group A (HMG A) proteins histone variant macroH2A and pRb. Appearance and functions of SAHF require an efficient p16ink4a - pRb pathway as p16ink4a inactivation prevents SAHF formation and Rb is recruited to genes that are associated with proliferation in order to repress them. This implicates active role of SAHF in transcriptional silencing of genes. Recent evidence suggests that SAHF are robustly detected in DNA damaging stimuli/oncogene induced senescence causing induction of H3K9me3, ATR/ATM and p16 activation, and S-phase arrest in parallel. Moreover, the total levels of histone acetylation are likely to change as the organism ages. Consistent with this observation, exogenous exposure to stress stimuli such as HDAC inhibitors Trichostatin A (TSA) or sodium butyrate incites a senescence-like state, suggesting that modulation of histone acetylation is a critical step in the establishment of senescence. Incidentally, nuclear chromatin irregularity has been one of the chief atypical changes observed in ovarian epithelium morphology speculating the epigenomic effects. Fig. 3a provides the epigenetic evidence to ovarian epithelial alterations educed by SIPS through increased SAHF formation and hypermethylation of H3K9me3 and hypoacetylation of H4 histone (Fig. 3b). Sustained expression of heterochromatin markers organized in sub-nuclear structures resembling SAHF have been indicated with early oncogenic events apart from stress induced senescence. We envisage that the observed SAHF, hypermethylated states of H3K9me3 and hypoacetylation of H4 in ovarian epithelial cells after MIC exposure have the putative role in the inception of phenotypic transformation (Fig. 3).

**Senescence associated secretory phenotype (SASP)**

The senescent phenotype does not merely represent the proliferation arrest of a cell. Instead, a senescent cell is a metabolically active phenotype that bears inestimable changes in protein expression and secretions such as interleukins (ILs), chemokines, growth factors and proteases, ultimately developing the senescence-associated secretory phenotype (SASP) or the senescence messaging secretome connecting the link between inflammation and cellular senescence. These studies demonstrated that senescence is associated with increased expression of inflammation-associated genes, including the chemokines monocyte chemotactic protein-1 (MCP-1) and Gro-α, cytokines IL-15 and IL-1β, Toll-like receptor 4 (TLR4) and intercellular adhesion molecule-1 (ICAM-1) similar to those observed in inflammatory responses and wound healing processes. In addition to replicative senescence, the induction of the inflammatory network is also linked to premature senescence induced by oncogenes (Ha-rasV12/ BRAFE600).

Another feature of the SASP is the duration that it takes for induction. Under in vitro conditions, cells develop a full SASP >5 days after senescence induction, and the cells’ growth arrests within 24 h of damage. Also not all SASP factors begin to be secreted at the same time since it is a gradual phenotypic transition feature conserved between cell types and senescence inducers.

SASP is known to influence the proliferation/differentiation of neighbouring cells and disrupt
tissue micro-architecture, principally through three major effects: (i) shedding of membrane associated proteins, resulting in soluble versions of membrane-bound receptors, (ii) cleavage/degradation of signaling molecules, and (iii) degradation or processing of the extracellular matrix. Rodier et al\textsuperscript{30} have shown that persistent DDR signaling proteins ATM and CHK2 are vital for the senescence associated secretion of inflammatory cytokines such as IL-6, IL-1, IL-8, and the tumour necrosis factors (TNF) are among the prominent cytokine of SASP. Through IL-6 expression, senescent cells can directly affect neighbouring cells that express the IL-6R (gp80) and gp130 signaling, which in turn act to trigger the nuclear factor kappa B (NF-κB) and activating protein 1 pathways\textsuperscript{46}. The progressive expression of NF-κB further lends evidence to the notion that that NF-κB signaling is the major pro-inflammatory feed-forward loop signaling pathway which stimulates the appearance of SASP\textsuperscript{47}.

miRNA in SIPS

Recent studies have shown that SIPS in human diploid fibroblasts and human trabecular meshwork cells is associated with upregulation of two microRNAs miR-182 and 183 from the miR-183-96-182 cluster. Overexpression of miR-182 leads to a significant increase in SA-β-galactosidase activity and might contribute to specific changes in gene expression associated with senescence\textsuperscript{48}. Further, it has been demonstrated that overexpressed miR-20a is able to induce premature senescence in mouse embryonic fibroblasts\textsuperscript{47}. On the other hand, miRNAs like mature let-7 target multiple genes, including architectural transcription factor HMGA2 which represses senescence markers p16 and p19 expression suggestive of antagonistic of senescence\textsuperscript{50}.

SIPS and chromosomal anomalies

Irreparable sustained DNA-damage checkpoint activation is postulated to be causally associated with the senescent state presenting extensive chromosomal abnormalities with irreversible damage at non-specific telomeric sites that serve as a signal for SIPS\textsuperscript{51}. Besides, defective DNA-damage response pathways might also result in chromosomal defects and/or premature cell senescence in culture, raising the possibility that these might initiate various phenotypes associated with chromosomal fragility syndromes\textsuperscript{52}. It is well known that increase in rate of gross chromosomal rearrangements frequently accelerates ageing\textsuperscript{53}. Fig. 4a demonstrates the SIPS mediated structural chromosomal anomalies such as chromatid breaks, fragmentation, Robertsonian p-p arm fusion, dicentric chromosomes, ring formation and tetraradial structures in mouse ovarian epithelial cells exposed to MIC at 48 h. These aberrations indicate the potential involvement of damage or loss of telomeric regions. The telomere-associated protein TRF2 (telomeric repeat binding factor 2) and POT1 (Protection of telomeres protein 1) are critical regulators of stress-induced telomere

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Fig. 3(a). SAHF formation. Representative microphotographs of B/CMBBA.Ov cells prior to and post-treatment with 0.005 μM MIC at 48 h showing punctate DAPI-stained DNA foci in nuclei depicting senescence associated heterochromatin foci (SAHF) and increased levels of heterochromatin marker tri-methylated histone 3 at lysine 20 (H3K9Me) which colocalised with focal nuclear staining for DAPI (blue). While there are no typical changes in corresponding control. (b) SIPS associated hypo-acetylation of histone H4. Cells were treated with 0.005 μM MIC for 48 and 96 h. Immuno-blotting with antibody against pan-acetylated H4 (AcH4) showed hypo-acetylation down the time points with maximum effect seen at 96 h. Blots represent mean of three independent experiments (n=3). β-actin was used as internal control.
Stress induced telomere attrition can accelerate senescence in mitotic cells and dysfunction of TRF2 results in the exposure of the telomere ends and activation of ATM-mediated DNA damage response to repair the chromosome ends, which would manifest in non specific chromosomal end joining and lead to devastating consequences for genomic integrity. Immuno-FISH study in treated mouse ovarian epithelial cells showed the loss of TRF2 expression at the chromosomal ends of treated cells at 48 h (Fig. 4b), thereby, fostering observations that telomeric ends are susceptible to the MIC exposure and resultant initiation of premature senescence. Spectral karyotype analysis of treated cells reveal the quadriradial formation due to translocation of chromosomes 1, 3 and 11(t1q; 3q; 11q) and also numerical gain of chromosome 15 and X chromosome with loss of chromosome 7 (Fig. 4c).

**Morphological and neoplastic transformation**

The factors secreted by senescent cells can alter the differentiation status of neighbouring cells. By virtue of these factors epithelial cells can undergo morphologic changes in culture that resemble an epithelial-to-mesenchymal transition in the presence of a senescent conditioned medium. Consequently such situation can lead to disruption of epithelial cell clusters and stimulate dedifferentiation in vitro and in vivo suggestive of pro-tumoural role of SASP in ageing epithelial cells. Studies have suggested that invaginations of the ovarian surface epithelium (OSE) into the stroma of the ovary as a result of inflammatory factors induced ovulation, endometriosis and pelvic inflammatory diseases, are associated with an increased risk for formation of epithelial inclusion cysts. As, TNF-α may enhance tumour growth and invasion by inducing the secretion of cytokines, pro-angiogenic factors, and metalloproteinases, this critical secretory factor of SASP is speculated to be involved in dysplastic changes in epithelial inclusion cysts, including transition of epithelial inclusion cysts from cuboidal to tall columnar ciliated epithelium, cellular stratification, loss of polarity, and epithelial tufting. Besides, downregulated expression of miRs let-7a, let-7b and miR-145, has been implicated in ovarian cancers. However, further characterization of these clones would divulge many more clues involved in neoplastic transformation in ovarian epithelial cells.

**Clinical translational perspective**

Cancer of the epithelia is the most common type, accounting for approximately 90 per cent of ovarian...
malignancies. The incidence and overall mortality of ovarian cancer have not changed appreciably in the past 50 years, this in large part, is due to an inadequate understanding of the early events in ovarian carcinogenesis. The notion that ovarian carcinoma originates in the OSE has been known for decades, and there seems to be a unanimity among investigators that this is in fact true. Majority of women diagnosed with this form of cancer already have an advanced stage of the disease, limiting treatment options and conveying a high risk of mortality. While most patients respond to initial chemotherapy and/or radiation treatments, approximately in 70 per cent of cases a recurrence has been observed. Therefore, a therapeutic intervention that could decrease tumour burden and increase sensitivity to available modalities of treatment would have a significant impact on the high mortality rate associated with ovarian cancer. Our results provide a detailed, quantitative insight into the role of SIPS in ovarian tumorigenesis. An important implication of our data could be the perturbation in mitochondrial-nuclear crosstalk via redox imbalance and altered inflammatory signaling, induced by environmental stress, which drives premature senescence and neoplastic transformation in the ovarian milieu. Our result substantiates that exogenous stress signals can deregulate the dynamic equilibrium of ovarian surface epithelium, and propounds an intriguing account of epigenetic dimension of SIPS, a hitherto unconnected molecular paradigm. We envisage that a detailed transcriptomic analysis of emerging daughter clones from the post-senescent mother phenotypes might help to discern novel molecular switches that regulate this stress-induced phenomenon.

Early detection and diagnosis of ovarian cancer still remains a major challenge. Although there are tests for women who are at high risk for the disease, including the CA-125 blood test, transvaginal ultrasound, and pelvic examination, there is still no reliable method available to detect the disease. To date, management of ovarian cancer has primarily focused on radical surgery, cytotoxic chemotherapy and/or radiotherapy, but never on strategies for molecular surveillance. We believe that a molecular risk-assessment approach has every potential to dramatically reduce the mortality and might prove beneficial in preserving fertility in young women and preventing loss of hormonal function in older women. More importantly, it can save women’s lives. Although translational utility of SIPS as a biomarker for estimating ovarian cancer risk seems evident from our studies, further investigations will be imperative.

![Fig. 5. Schematic model. A novel hypothesis depicting the possible role of stress induced premature senescence (SIPS) in ovarian tumorigenesis, orchestrated through genomic and epigenomic level alterations. ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia Rad3 related; p16 INK4, p16 inhibitor of cyclin dependent kinase 4; Rb, retinoblastoma; CDCK2, cyclin dependent kinase 2; E2F, elongation 2 promoter binding factor; SA-β-gal, senescence associated beta galactosidase, SAHF, senescence associated heterochromating foci, SASP, senescence associated secretory phenotype.](image-url)
to provide a tangible way for its precise validation in clinical settings.

Acknowledgment

This work was supported by a Young Investigator Award grant to the second author (PKM) from the Department of Biotechnology, Ministry of Science & Technology, Government of India, New Delhi.

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