PKC\(\eta\) promotes senescence induced by oxidative stress and chemotherapy

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Senescence is characterized by permanent cell-cycle arrest despite continued viability and metabolic activity, in conjunction with the secretion of a complex mixture of extracellular proteins and soluble factors known as the senescence-associated secretory phenotype (SASP). Cellular senescence has been shown to prevent the proliferation of potentially tumorigenic cells, and is thus generally considered a tumor suppressive process. However, some SASP components may act as pro-tumorigenic mediators on premalignant cells in the microenvironment. A limited number of studies indicated that protein kinase C (PKC) has a role in senescence, with different isoforms having opposing effects. It is therefore important to elucidate the functional role of specific PKCs in senescence. Here we show that PKC\(\eta\), an epithelial specific and anti-apoptotic kinase, promotes senescence induced by oxidative stress and DNA damage. We further demonstrate that PKC\(\eta\) promotes senescence through its ability to upregulate the expression of the cell cycle inhibitors p21\(^\text{Cip1}\) and p27\(^\text{Kip1}\) and enhance transcription and secretion of interleukin-6 (IL-6). Moreover, we demonstrate that PKC\(\eta\) creates a positive loop for reinforcing senescence by increasing the transcription of both IL-6 and IL-6 receptor, whereas the expression of IL-8 is specifically suppressed by PKC\(\eta\). Thus, the presence/absence of PKC\(\eta\) modulates major components of SASP. Furthermore, we show that the human polymorphic variant of PKC\(\eta\), 374I, that exhibits higher kinase activity in comparison to WT-374V, is also more effective in IL-6 secretion, p21\(^\text{Cip1}\) expression and the promotion of senescence, further supporting a role for PKC\(\eta\) in senescence. As there is now considerable interest in senescence activation/elimination to control tumor progression, it is first crucial to reveal the molecular regulators of senescence. This will improve our ability to develop new strategies to harness senescence as a potential cancer therapy in the future.

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Cellular senescence refers to permanent cell-cycle arrest, resulting in stable and long term loss of proliferative capacity, despite continued cell viability and metabolic activity. Senescence was initially identified as the process that limits the replicative life span of cultured human cells, due to the gradual loss of telomeric DNA at the ends of chromosomes (replicative senescence), generating a persistent DNA damage response (DDR).\(^1\) However, senescence can also be induced in the absence of detectable telomere loss or dysfunction by various cellular and environmental stressors, such as culture shock, ionizing radiation or prolonged exposure to substantial doses of oxidative stress, generally known as stress-induced premature senescence.\(^2\) The definition of senescence was broadened to include oncogene-induced senescence also known as stress or aberrant signaling induced senescence.\(^3\) Cellular senescence appears to be an anti-proliferative process that limits the progression of damaged cells. Thus, in addition to acting as a potent barrier to tumorigenesis, senescence contributes to the cytotoxicity of certain anti-cancer agents, thereby dictating the outcome of chemotherapy treatment.\(^4,5\) Evidence of the existence of premature senescence in vivo has accumulated, supporting a role for senescence in tumor suppression. For example, naevi on human skin were shown to contain oncogenic mutations, have undergone senescence and therefore failed to develop into malignant tumors.\(^6\)

Senescent cells have characteristic features, displaying a large and flat morphology, an increase in senescence-associated \(\beta\)-galactosidase (SA-\(\beta\)-gal) activity,\(^7\) and in some cell types, a distinct change in chromatin organization known as senescence-associated heterochromatin foci.\(^8\) In addition to executing a cell-cycle arrest program, senescent cells undergo massive changes in the expression of genes thought to influence the tissue microenvironment in vivo. Thus, in addition to repressing genes related to proliferation, senescent cells often secrete inflammatory cytokines and immune modulators, downregulate extracellular matrix proteins and upregulate enzymes that degrade extracellular matrix, resulting in the senescence-associated secretory phenotype (SASP) (also called the senescence-messaging secretome\(^9\)). The senescence secretome has tumor suppressive effects, inducing growth arrest and stimulating the immune system to

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**Abbreviations:** CDK, cyclin-dependent kinase; DDR, DNA damage response; H2AX, H2A histone family, member X; IGFBP, insulin-like growth factor-binding protein; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; JNKs, c-Jun N-terminal kinases; MARCKS, myristoylated alanine-rich C-kinase substrate; MBP, myelin-basic protein; NF\(\kappa\)B, nuclear factor kappa B; PKC, protein kinase C; Rb, retinoblastoma; SA-\(\beta\)-gal, senescence-associated \(\beta\)-galactosidase; SASP, senescence-associated secretory phenotype; TNFa, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor

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clear senescent cells. However, components of the secretome can induce pro-tumorigenic effects on premalignant cells in the microenvironment. Several non-cell-autonomous activities of SASP show the functional relevance of senescence in pathophysiology, in particular in the tumor microenvironment, enhancing tumorigenesis of neighboring cells and playing a role in the decline of organ function with aging.

Several studies indicated that protein kinase C (PKC) members have a role in senescence, sometimes exhibiting opposing effects. It was also suggested that individual PKCs trigger distinct responses when activated in different phases of the cell cycle via a common mechanism that involves the cyclin-dependent kinase (CDK) inhibitor p21Cip1. PKCδ was shown to be involved in activating senescence in primary diploid cells. Activation of PKCa in lung cancer cells during the S phase of the cell cycle led to G2/M arrest and cellular senescence, an effect that involved p21Cip1 upregulation and irreversible inhibition of cell proliferation. As these lung cancer cells do not express p16INK4a or p53, the upregulation of p21Cip1 was p53 independent. While PKCa enhanced senescence, activation of the atypical PKCδ isoform suppressed senescence in breast and glioblastoma cells. PKCδ depletion led to an increased number of senescent cells, showing no requirement for p53, p16INK4a or ARF, but was markedly dependent on p21Cip1.

Here we show that the novel PKC isoform, PKCη, promotes senescence induced by oxidative stress and DNA damage. The PKCη isoform has a unique tissue distribution and is primarily expressed in epithelial tissues and in cells with high turnover. PKCη was found to be involved in diverse cellular functions including terminal differentiation, proliferation and secretion. The mechanism of action described in some of these studies involved modulation of cell-cycle components and a role in cell cycle progression at the G1 to S phase. PKCη was shown to activate a cellular program that includes increased expression of cyclin E, as well as the induced expression of the cyclin-dependent kinase inhibitor p21Cip1. Moreover, PKCη formed a complex with Cdk2 and cyclin E in the perinuclear region and was shown to phosphorylate p21Cip1.

Recent studies suggest that PKCη plays a special role in the response to stress and regulation of apoptosis. It provides protection against apoptosis induced by chemotherapy drugs in Hodgkin’s lymphoma cell lines and breast adenocarcinoma MCF-7 cells. In addition, PKCη expression was found to correlate with drug resistance and drug-resistance associated genes in patients with breast cancer, ovarian cancer and AML blasts. PKCη expression and membrane localization were shown to be markers for a poor prognosis in lung and breast cancers, respectively.

Notably, the PKCη gene (PRKCH) was recently identified as a risk factor for cerebral infarction. Its gene polymorphism, including a SNP in the coding region (changing valine to isoleucine at position 374 of the kinase domain), was also associated with increased susceptibility for rheumatoid arthritis in Japanese and Chinese populations, as well as a risk of severe gastric atrophy.

Here we show that PKCη promotes senescence induced by oxidative stress and DNA damage via its ability to upregulate the expression of the cell cycle inhibitors p21Cip1 and p27Kip1, and create a positive loop for reinforcing senescence by enhancing the expression of both interleukin-6 (IL-6) and its receptor. Notably, the expression of IL-8, another major component of SASP, was specifically suppressed by PKCη expression, further demonstrating its ability to modulate SASP. Moreover, we show that in comparison to WT-374V, the polymorphic variant 374I of PKCη exhibits enhanced kinase activity, increased secretion of IL-6, enhanced expression of p21Cip1 and higher levels of senescence, further supporting a role for PKCη in senescence.

Results

PKCη expression enhances markers of senescence upon oxidative stress and chemotherapy-induced DNA damage. One of the characteristic markers of senescent cells is an increase in senescence-associated β-galactosidase. Oxidative stress and chemotherapy drugs, such as etoposide and doxorubicin were previously shown to induce senescence in MCF-7 cells. MCF-7 cells expressing sh-PKCη (clones 2-2, 3-3) and scrambled control cells (clone 5-3) were treated with H2O2 or etoposide under conditions that induce senescence. As seen in Figure 1, in response to H2O2 or etoposide, PKCη-knockdown cells showed significantly and reproducibly lower number of SA-β-galactosidase-stained cells compared with scrambled control cells (Figures 1a and b). Similar results were obtained with doxorubicin (data not shown).

Senescence is characterized by permanent irreversible cell-cycle arrest. Therefore, we assessed the number of proliferating cells after the implementation of senescence. As shown in Figures 1c and d a lower number of proliferating cells were regrown out of senescent cultures of scrambled (sh scr 5-3) control cells compared with PKCη-knockdown (sh 3-3 and sh 2-2) cells in colony formation assays. Viability assays (Neutral Red) with additional PKCη-knockdown clones (sh 4-2 and sh 3-5) showed similar results (data not shown). Thus, these results most likely reflect the higher percentage of senescent cells that did not proliferate in PKCη-expressing scrambled (sh scr 5-3) control cells. Several PKCη-knockdown clones were employed in the experiments presented here as these clones lose their silencing abilities over time. Nonetheless, silencing was verified in each experiment for each clone.

The fact that proliferation is blocked in senescence is accompanied by the upregulation of cell cycle control genes. Different studies have indicated that the cell cycle inhibitor p21Cip1 is required to induce senescence. Our results showed increased expression of p21Cip1 and p27Kip1 in PKCη-expressing cells (sh scr 5-3), which was markedly reduced in PKCη-knockdown cells upon etoposide treatment (Figure 2). Cell-cycle arrest upon etoposide treatment was verified by decreased phosphorylation of the retinoblastoma (Rb) protein compared with untreated control cells. In addition, DcR2, another senescence marker, was elevated in PKCη expressing cells (Figure 2a).

Etoposide is known to cause DNA damage, triggering DDR, which is one of the mechanisms underlying senescence. γ-H2A histone family, member X (γ-H2AX) phosphorylation at
PKCζ promotes senescence upon oxidative stress and etoposide-induced DNA damage. (a) MCF-7 clones expressing sh-PKCζ (sh η 3-3 and sh η 2-2) or scrambled control cells (sh scr 5-3) were treated with 150 μM H2O2 or 400 μM etoposide for 2 h. Fresh medium was added for 96 h. Cells were stained both for Hoechst and SA-β-Gal as described in Materials and Methods. The total cell number was determined by nuclear fluorescent staining. (b) SA-β-gal representative staining of sh-PKCζ and scrambled control clones are shown (original magnification × 100 and × 400 for untreated and treated cells, respectively). (c) The untreated control cells and the H2O2-treated senescent cultures of the different clones (sh scr 5-3, sh η 3-3 and sh η 2-2) were replated (1 × 10⁵ cells of each clone) and grown in fresh medium. Cell proliferation was determined using the colony forming assay as described in Materials and Methods. (d) Shown is the quantification of the number of colonies stained by crystal violet shown in c. Results are the average of at least three experiments. Error bars represent the S.D.; two-tailed, unpaired sample t-test statistical analysis is shown: * indicates statistical significance compared with untreated cells of the same clone, † indicates statistical significance compared with other clones. *P ≤ 0.05, **P ≤ 0.001, ^^^P ≤ 0.0001
Ser139 is a marker for DNA breaks, initiating DDR. Our results show increased phosphorylation of H2AX in PKC\(\eta\)-knockdown cells under senescence-inducing conditions, triggered by etoposide (Figure 2a) or \(H_2O_2\) (Figure 2b). The phosphorylation of \(\gamma-H2AX\) may reflect the extent of DNA breaks but also the cellular response to DNA damage. Therefore, to directly evaluate the extent of DNA breaks, we performed comet assays (Figures 2c and d). Our results show similar levels of initial DNA damage after 2 h; however, after 96 h, lower numbers of cells exhibiting DNA breaks were observed in PKC\(\eta\)-knockdown cells, suggesting that the response to DNA damage (repair processes) was more efficient in these cells.

**PKC\(\eta\) exhibits opposing effects on IL-6 and IL-8 transcription and secretion: it enhances IL-6 but suppresses IL-8.** Senescence is accompanied by enhanced secretion of cytokines, growth factors and proteases-collectively defining the SASP. Components of the senescence secretome reinforce or implement cell-cycle arrest and contribute to tumor suppression by signaling and recruiting the immune system to eliminate senescent cells. Among these, IL-6 secretion was implicated in reinforcing senescence\(^5,40\) but also in promoting tumor invasiveness.\(^41\)

We have initially determined the transcription of cytokines in PKC\(\eta\)-knockdown cells using RT-PCR in response to oxidative stress and chemotherapy (Figure 3). mRNA levels of all cytokines examined, that is, IL-6, IL-8, tumor necrosis factor alpha (TNF-\(\alpha\)), vascular endothelial growth factor (VEGF) and insulin-like growth factor-binding protein (IGFBP-7), were increased, demonstrating their induction in senescence (data not shown). However, PKC\(\eta\)-knockdown had an effect on IL-6 and IL-8 (Figure 3) but not on the transcription of other cytokines. Oxidative stress markedly increased IL-6 mRNA expression in scrambled control cells (sevenfold) compared with its increase in PKC\(\eta\)-knockdown cells (twofold). Etoposide had a similar effect on IL-6 expression (Figure 3a). Notably, PKC\(\eta\)-knockdown cells exhibited higher levels of IL-8 secretion in response to oxidative stress and chemotherapy (30–40-fold increase) compared with PKC\(\eta\)-expressing scrambled control cells (10-fold increase). Thus, our studies suggest that PKC\(\eta\) is a negative regulator of IL-8 transcription (Figure 3b).

Next, we have examined whether the opposite effects of PKC\(\eta\) on the transcription of IL-6 and IL-8 are consistent with
PKCη promotes senescence

U Zurgil et al

Cell Death and Disease

The polymorphic variant V374I enhances the kinase activity of PKCη, IL-6 production, p21Cip1 expression and senescence. The PKCη polymorphic variant, 374I, in which valine replaces isoleucine at position 374 in its kinase domain (Figure 6a), was previously implicated in an increased risk for brain infarction. A plasmid containing this polymorphic variant was introduced into Cos-7 and MCF-7 cells (Figure 6). Results showed that this polymorphic variant exhibited significantly enhanced kinase activity, both in autophosphorylation assays and when myelin-basic protein (MBP) or myristoylated alanine-rich C-kinase substrate (MARCKS) were used as exogenous substrates (Figures 1b and c).

We then examined whether this polymorphic change would affect the secretion of IL-6. Indeed, the variant V374I exhibited increased IL-6 secretion when expressed in MCF-7 cells compared with WT-374V (Figure 6e). This could not be the result of higher 374I expression, since 374I and 374V were expressed equally (Figure 6d).

Our present studies, using sh-RNA PKCη-knockdown cells, showed that PKCη had a role in the enhanced expression of the cell cycle inhibitor, p21Cip1 (Figure 2). Here we show that overexpression of both 374V and 374I in MCF-7 cells increased the expression of the senescence marker p21Cip1 under oxidative stress conditions, with the polymorphic variant 374I having a greater effect (Figure 6d). The mutation of the INK4a/ARF locus in MCF-7 cells may explain the weak effect observed in p16INK4a expression in these cells. Phosphorylation of Rb protein was decreased under oxidative stress conditions, consistent with the fact that these cells stopped proliferating. Furthermore, the numbers of β-gal-positive cells detected in cells overexpressing the 374I variant were higher compared with that of WT or empty vector transfected cells (Figure 6f). Taken together, our studies suggest that the kinase activity of PKCη is important in promoting senescence and its maintenance.

Discussion

Cellular senescence appears to be an important mechanism preventing the proliferation of potential cancer cells. The senescence program impacts the senescence-associated cell-cycle arrest and the tissue microenvironment. Here we show that PKCη promotes senescence induced by oxidative stress and chemotherapy via two mechanisms; one is the upregulation of p21Cip1 and p27kip1 that are crucial for cell cycle inhibition. The other is the increased secretion of both IL-6 and its receptor, forming a positive loop for enhanced IL-6 secretion, which could establish and further reinforce the

Figure 3  PKCη expression has opposing effects on IL-6 and IL-6 transcription in response to oxidative stress and DNA damage. MCF-7 clones expressing sh-PKCη (sh2-2 and sh3-3) or scrambled (sh5-3) control cells were treated with 150 μM H2O2 or 400 μM etoposide for 2 h, followed by the addition of fresh medium for 96 h. Total RNA was extracted and cDNA was generated by real-time PCR according to the manufacturer’s instructions. (a) IL-6 mRNA and (b) IL-8 mRNA levels are depicted. Columns represent mean ± S.E. of three independent experiments (* indicates statistical significance compared with untreated cells, # indicates statistical significance compared with other clones). Two-tailed, unpaired sample t test statistical analysis is shown: tP ≤ 0.05, **tP ≤ 0.001 and ***tP ≤ 0.0001.
senescence phenotype. The role of PKC\(\eta\) in enhancing IL-6 secretion while inhibiting IL-8 secretion could have important implications on the recruitment of immune cells to the senescent microenvironment. Hence, PKC\(\eta\) could modulate secretome properties. Moreover, the role of PKC\(\eta\) in promoting senescence is further demonstrated by employing its 374I polymorphic variant (in the ATP binding domain). This polymorphic variant, exhibiting higher kinase activity compared with WT -374V, was also more effective in promoting the secretion of IL-6, increasing p21 Cip1 and augmenting senescence.

Senescent cells express a SA-\(\beta\)-gal which partly reflects the increase in lysosomal mass. Our results show that the percentage of senescent cells identified by \(\beta\)-gal staining in response to oxidative stress or chemotherapy-induced senescence was significantly diminished when PKC\(\eta\) was knocked down. Moreover, re-plated senescent cultures of PKC\(\eta\)-knockdown cells exhibited higher proliferation and colony formation capacity compared with PKC\(\eta\)-expressing control cells (Figures 1b–d), in accordance with a smaller fraction of senescent cells present in the PKC\(\eta\)-knockdown cells.

Cellular senescence pathways are believed to have multiple layers of regulation. Among the cellular pathways reported to regulate senescence are the p16INK4a/pRB pathway, the p19ARF/p53/p21CIP1/WAF1 pathway and the PTEN/ p27KIP1 pathway.\(^{45}\) p21Cip1 has direct inhibitory actions on the cell-cycle machinery and correlates well with declining growth rates in cultures undergoing senescence. Indeed, induction of p21Cip1 causes cell-cycle arrest in senescent cells.\(^{46}\) p27kip1 was previously shown to promote senescence in multiple tissues and loss of p27kip1 expression led to downregulation of senescence and progression of cancer.\(^{47,48}\) Moreover, an intrinsic cooperation between p21Cip1 and p27kip1 in the activation of stress-induced senescence and tumor progression was demonstrated in vivo.\(^{49}\)

According to our previous studies, PKC\(\eta\) has a role in cell cycle regulation. The expression of the cyclin-dependent kinase inhibitor p21Cip1 was specifically elevated in PKC\(\eta\)-expressing cells.\(^{20}\) In addition, p21Cip1 was associated with the cyclin E/Cdk2 complex in cells under stress of serum starvation.\(^{22}\) Moreover, p21Cip1 was shown to associate with the PKC\(\eta\)/Cyclin E/Cdk2 complex in keratinocytes and to be phosphorylated by PKC\(\eta\).\(^{21}\) The functional consequence of this phosphorylation has not been elucidated.

Our results presented here suggest that PKC\(\eta\) plays a role in the upregulation of p21Cip1 and p27kip1 in senescence, as we show a marked decrease in p21Cip1 and p27kip1 expression in PKC\(\eta\)-knockdown cells (Figure 2). Although PKC\(\eta\) increased p21Cip1 expression, it had no effect on p53 levels in treated cells (data not shown), suggesting that the upregulation of p21Cip1 is p53 independent. Further studies should be conducted to determine whether p21Cip1 is directly phosphorylated by PKC\(\eta\) under conditions that lead to senescence. Decreased p-AKT activity was shown to promote cellular proliferation under conditions of stress.

**Figure 4** PKC\(\eta\) expression has opposing effects on IL-6 and IL-8 secretions. MCF-7 clones expressing sh-PKC\(\eta\) (sh \(\eta\) 3-5, sh \(\eta\) 2-2 and sh \(\eta\) 4-2) or scrambled control cells (sh scr 5-3) were treated with 150 \(\mu\)M H\(_2\)O\(_2\) or 400 \(\mu\)M etoposide for 2 h. Fresh growth medium was added for 96 h. Medium was collected and assayed by ELISA for (a, b) IL-6 and (c, d) IL-8 protein levels as described in Materials and Methods. Values are represented as pg/ml, calculated from standard curves of at least three separate experiments performed in triplicate. Error bars represent the S.D.; two-tailed, unpaired sample t-test statistical analysis is shown: *\(P \leq 0.05\), **\(P \leq 0.001\) and ***\(P \leq 0.0001\) (* represents statistical significance for each non-treated and treated clone and \(*\) represents statistical significance between different clones).
PKCζ promotes senescence

U Zurgil et al

DNA damage via inhibition of c-Jun N-terminal kinases (JNK) activity.26 H2AX was found to be a target of the JNK signaling pathway,24 which could explain its reduced phosphorylation in PKCζ-expressing control cells. Direct analysis of double strand DNA breaks by the comet assay showed more DNA breaks in PKCζ-expressing control cells subsequent to initial equal DNA damage (Figures 2c and d), suggesting a reduced cellular response to DNA damage in PKCζ-expressing control cells. This point needs further investigation.

Senescent cells secrete a complex mixture of extracellular proteins and soluble factors, known as the SASP.55 nuclear factor kappa B (NFκB) was shown to be a master regulator of SASP, by influencing the expression of NFκB target genes. Proteomic analysis of senescent chromatin identified the NFκB subunit p65 as a major transcription factor that accumulates on chromatin of senescent cells.56 We have recently reported that PKCζ regulates NFκB upstream signaling by activating the IkB kinase and the degradation of IkB. Furthermore, PKCζ enhanced nuclear translocation and transactivation of NFκB under non-stressed conditions and in response to DNA damage by camptothecin.24 Thus, PKCζ could exert its effects on SASP through its role in NFκB activation in these cells.24

Components of the senescence secretome reinforce cell-cycle arrest and contribute to tumor suppression by signaling and recruiting components of the immune system10. Here we demonstrate that PKCζ expression modulates the senescence secretome, mainly by inducing the transcription and secretion of IL-6 and suppressing IL-8 (Figures 3 and 4). Although the mRNA levels of other SASP components, such as TNFα, IGFBP-7 and VEGF, were induced upon oxidative stress or chemotherapy, their expression was not affected by PKCζ (data not shown). IL-6 was found to be a key factor in generating cell-cycle arrest characteristics of senescence.5,57 Moreover, IL-6 and its receptor, IL-6Ra, form a senescence-inducing circuit.42 For example, IL-6 was reported to act in concert with its receptor to induce cell-cycle arrest in response to BRAF oncogenic stress.11 Our studies show that the overexpression of PKCζ in MCF-7 cells induced the expression of both IL-6 and IL-6R (Figure 5), suggesting that this circuit could be the underlying mechanism for the enhanced IL-6 expression in PKCζ expressing cells in senescence.

PKCζ expression resulted in the suppression of IL-8 mRNA transcription and secretion, which was alleviated when PKCζ was knocked down (Figures 3 and 4). Secretion of IL-8 was frequently detected in senescent cells; however, in contrast to IL-6, its specific contribution to senescence has not been established.58 The expression of IL-8 in breast cancer cell lines was shown to require a complex cooperation between NFκB, AP-1 and C/EBP transcription factors.59 Binding sites for these transcription factors were identified in the IL-6 promoter region.60 Although NFκB is crucial for IL-8 gene transcription, its cooperation with either AP-1 or C/EBP is required to fully activate IL-8 transcription in breast cancer cells.59 Moreover, it was demonstrated that IL-6 expression is regulated by NFκB, while the regulation of IL-8 more closely correlated with AP-1 activity.61 Several studies demonstrated that inhibition of PKC blocks NFκB and AP-1 activity, suggesting direct regulation of these transcription factors by PKC.62 Furthermore, IL-8 release was downregulated by

senescence through upregulation of p53 and p27Kip1 expression.50 We have previously shown that activated PKCζ reduces phosphorylation of AKT induced by insulin-like growth factor 1,26 which could be responsible for the increase in p27Kip1. Alternatively, the increase in p21Cip1 and p27Kip1 could result from the effects of PKCζ on the transcription and secretion of IL-6 shown here (Figures 3,4,5,6), since IL-6 was previously shown to upregulate p27Kip1 and p21Cip1, leading to cell-cycle arrest.51,52

Various stimuli inducing senescence often activate DDR pathways involved in both the induction and maintenance of senescence.53 Our results show reduced γ-H2AX phosphorylation, one of the DDR markers, in senescent cultures of PKCζ-expressing cells (Figure 2). We have previously reported that PKCζ protects against cell death induced by

![Figure 5](image)

*Figure 5* Overexpression of PKCζ in MCF-7 cells increases transcription of IL-6 and IL-6R upon DNA damage. MCF-7 cells were transfected with PKCζ cDNA and the control vector pHACE. The cells were treated with 2 μM doxorubicin for 2 h, followed by fresh medium for 48–96 h. Total RNA was extracted and cDNA was generated by reverse transcription as described in Materials and Methods. RNA products were analyzed by real-time PCR according to the manufacturer’s instructions. (a) IL-6 mRNA and (b) IL-6R mRNA levels are depicted. Columns represent mean ± S.E. of three independent experiments (* indicates statistical significance compared with untreated cells, **indicates statistical significance compared with other clones). Two-tailed, unpaired sample t-test statistical analysis is shown: ^P ≤ 0.05, ***P ≤ 0.001 and ****P ≤ 0.0001.
inhibition of JNK activity.61 Our results demonstrating that PKCη is a repressor of IL-8 are in accordance with the notion that AP-1 is important for its regulation, since we have previously shown that PKCη inhibits JNK activity.26 Thus, in the absence of PKCη, JNK will enhance the expression of IL-8 through AP-1 binding and activation of the IL-8 promoter. IL-8 is a proinflammatory molecule that functions within the tumor microenvironment. It acts on leukocytes and endothelial cells, via their CXCR1 and CXCR2 receptors to promote immune infiltration and angiogenesis.58 Furthermore, signaling through CXCR2 leads to senescence in p53-WT and non-transformed cells.57

In summary, our present study demonstrates that PKCη has a role in establishing the senescence phenotype and the presence or absence of PKCη has a profound effect on SASP. PKCη expression suppresses the expression of IL-8 but activates a positive loop for IL-6 expression. Hence, PKCη reinforces senescence through an IL-6 dependent autocrine mechanism, while the suppression of IL-8 could affect the microenvironment in a paracrine manner. As there is now considerable interest in developing novel therapies to activate senescence to control tumor promotion, or therapies to eliminate senescence, it is crucial to first elucidate the molecular regulators of senescence. This will improve our understanding of the role of PKCη in cancer biology.
ability to develop new strategies to harness senescence as a potential cancer therapy in the future.

Materials and Methods

Cell culture, antibodies, reagents and transfections. Cells were grown in Dulbecco’s Modified Eagle Medium containing 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine and 10% Fetal Bovine Serum in a 5% CO2 humidified atmosphere at 37 °C. PKCγ-shRNA-expressing, stable MCF-7 derived cell lines were generated using the SureSilencing (SA Biosciences, Frederick, MD, USA) pre-designed PKCγ short hairpin RNA plasmids (shPKCγ 4-2, shPKCγ 2-2, shPKCγ 3-3 and shPKCγ 3-5) or a scrambled control vector (sh scramble 5-3).

Antibodies included: anti-PKCI (sc-215), anti-p21Cip1 (sc-397), anti-p16 (sc-688), anti-DR2 (sc-7505), which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-actin (691001, ICN Biomedicals, Irvine, CA, USA), anti-β1 (clone 16E12 Biononocul (MPSM-101P, Convance, Emeryville, CA, USA), anti phospho-Akt (Santa Cruz, CA, USA), anti-phospho-AKAP79 (Santa Cruz, CA, USA), anti-phospho-Akt (Santa Cruz, CA, USA), anti-p21 (Santa Cruz, CA, USA), anti-p16 (Santa Cruz, CA, USA), anti-p38 (Santa Cruz, CA, USA), anti-p53 (Santa Cruz, CA, USA), anti-EGFR (Santa Cruz, CA, USA), anti-apoptosis signal-regulating kinase 1 (ASK1) (Santa Cruz, CA, USA), anti-β-actin (IBA001, Sigma-Aldrich, St. Louis, MO, USA) or CST-MARKCS was added to the beads before the reaction mix was used. [γ-32P]ATP 4Ci/mmol was added to the mix seconds before initiating the reaction. All solutions were prepared on ice. Samples containing 20 μl beads were mixed with 23 μl kinase assay mix and incubated for either 10 or 20 min at 30 °C. The reaction was stopped by adding 10 μl sample buffer and 5 min denaturation at 95 °C, followed by spin down. All samples were run on 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Herts, UK). Kinase activity was evaluated using a phosphor-imager (Personal Molecular Imaging (PMI) System, Bio-Rad) and developing film exposures.

β-galactosidase staining. SA-β-gal activity was determined using a previously described protocol with some modifications. Briefly, cells were washed once with PBS, fixed with 0.5% glutaraldehyde (PBS (pH 7.2)), and washed in PBS (pH 7.2) supplemented with 1 mM MgCl2. Cells were stained in X-gal solution (1 mg/ml X-gal (Boehringer, Ingelheim, Germany), 0.12 mM K3Fe(CN)6, 0.12 mM K4Fe(CN)6, 1 mM MgCl2, in PBS at pH 6.0) overnight at 37 °C. Cells were photographed using an IX70 Olympus optical light microscope (Olympus, Tokyo, Japan). To estimate total cell numbers, cell cultures were stained with Hoechst 10 mg/ml (Calbiochem, HO 33342) for 30 min at 37 °C prior to β-gal staining. Hoechst fluorescence was detected using a light source providing light at 340–380 nm, emission was at 465 nm. SA-β-gal-positive cells were calculated as the percentage of Hoechst-stained cells.

Cytokine analysis. MCF-7 cells, transfected with PKCγ shRNA expressing plasmids, were grown in six-well plates (4 × 105 cells/well). After 24–48 h, the cells were treated with 150 μM H2O2, 400 μM etoposide (Ebewe Pharma, Unterach, Austria) or 2 μM doxorubicin for 2 h (as described above). Supernatants were collected from the culture medium. Cytokine levels were evaluated using the manufacturer’s standard curves. The ELISA kits used were: IL-6 and IL-8 (Cat. # 88-7068-88 and 88-8068, eBioscience, San Diego, CA, USA).

mRNA isolation, cDNA synthesis and quantitative real-time PCR. Total RNA was isolated and purified using an RNA extraction kit (5Prime, # 2900319, PerfectPure, Gaihurbs, MD, USA) according to the manufacturer’s protocol. The carried-over DNA was eliminated by treatment with DNase (Turbo DNA-free, Ambion, Foster City, CA, USA). The first-strand cDNA was reverse transcribed from 0.5–1 μg total RNA using the Reverse-IT first-strand synthesis kit and random hexamer primers (ABgene, Surrey, UK). Quantitative real-time PCR was performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Specific mRNA levels were quantified using SYBR green reagents (Kapa Biosystems, Cape Town, South Africa): primers for IL-8 (5′-GAA GCCGCCAGAAGACAACA-G3′ (forward) and 5′-AGAGCCTGTCGGACAGAAGC-3′ (reverse)); TNFα (5′-ATCTGGGATGATCGGGC-3′ (forward) and 5′-TTTGGT GAACAACCTGAAAC-3′ (reverse)); IL-6 (5′-GGGCTGAAGCTGAAAGCA-G3′ (forward) and 5′-GAGTTACAGTGTGACGTCG-3′ (reverse)); and VEGF (5′-CTCCGATGGAGTTCCTGTG-3′ (reverse)). The expression of these genes was normalized to 18S ribosomal RNA (18S rRNA) content using the ΔΔCT method. Statistical analysis of differences between the groups was performed as described below.

Cell death and disease

Colony formation assays. Cells treated for senescence induction were harvested with trypsin-EDTA and counted using a hemocytometer. A glass Pasteur pipette was used to ensure cell separation. Cells were diluted in complete medium and, 1 × 104 cells were plated in 200 mm tissue culture dishes. After incubation for 10 ± 1 days in high glucose media, 10% Fetal Bovine Serum and 10% Penicillin/Streptomycin at 37 °C, 5% CO2 and 95% Relative Humidity, the cells were washed with PBS and stained with 0.5% Crystal Violet in glutaraldehyde for 30 min at room temperature. Culture dishes were submerged and washed in tap water three to five times. Plates were left to dry overnight. The size and density of visible colonies were evaluated.

 Comet assay. PKCγ shRNA stable cell lines of PKCγ knockdown cells or scrambled control cells were seeded in six-well plates (4 × 105 cells/well). After 24–48 h, the cells were treated with 400 μM etoposide for 2 h, followed by replacement with fresh medium for 96 h. Single-cell gel electrophoresis was performed as described. Briefly, fully frosted microscope slides were covered with 150 μl of 15% normal melting-point agarose in PBS and then flattened using a cover slip for 5–10 min at 4 °C. The dried slides were kept at room temperature. Approximately
5 × 10^5 cells per sample (10 μl) were mixed with 0.5% (w/v) low melting agarose (120 μl) to form a cell suspension, which was then pipetted onto the first agarose layer, flattened and solidified (see above). After removal of the cover slip, the slides were immersed in the neutral lysis solution at 4 °C for 1 h or longer in the dark. The volume of comet tails of at least 250 cells per time point was assessed.

Statistical analysis. The statistical significance of differences between experimental groups was determined using the ANOVA test of variance in cases statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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