HIF1α delays premature senescence through the activation of MIF

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Premature senescence in vitro has been attributed to oxidative stress leading to a DNA damage response. In the absence of oxidative damage that occurs at atmospheric oxygen levels, proliferation of untransformed cells continues for extended periods of time. We have investigated the role of the hypoxia-inducible factor 1α (HIF1α) transcription factor in preventing senescence in aerobic and hypoxic conditions. Using embryonic fibroblasts from a conditional HIF1α knockout mouse, we found that loss of HIF1α under aerobic conditions significantly accelerated the onset of cellular senescence, and decreased proliferation under hypoxia. Furthermore, we identify the macrophage migration inhibitory factor (MIF) as a crucial effector of HIF1α that delays senescence. Inhibition of MIF phenocopies loss of HIF1α. Our findings highlight a novel role for HIF1α in preventing senescence under aerobic conditions, and identify MIF as a target responsible for this function.

Keywords: HIF1α; MIF; senescence; hypoxia; oxidative stress

RESEARCH COMMUNICATION

Cellular senescence has emerged as a programmed cellular stress response that is induced due to the accumulation of damage to a cell. Whether through the shortening of telomeres associated with a high number of cell divisions, activation of oncogenes, or DNA damage due to oxidative stress, induction of senescence in primary cells leads to an irreversible arrest phenotype that is characterized by the activation of the p53 and Rb proteins, as well as extensive chromatin modifications associated with the silencing of S-phase-promoting genes (Narita et al. 2003). In this way, senescence can be seen as a tumor suppressor mechanism that prevents excessive cellular divisions, or division of damaged cells (Ben-Porath and Weinberg 2005).

There is increasing evidence that senescence plays a critical role as a tumor suppressor in vivo. Senescent cells have been found recently in early-stage human prostate cancer specimens and premalignant melanocytic nevi, as well as in experimental models of lung adenocarcinoma and Ras-driven lymphoma (Braig et al. 2005; Chen et al. 2005; Collado et al. 2005; Michaloglou et al. 2005). These findings not only substantiate the significance of in vitro models of senescence, but also suggest novel therapeutic avenues aimed at reinitiating senescent programs in malignant cells (Lowe et al. 2004; Sharpless and DePinho 2005).

In normal tissue culture conditions, murine embryonic fibroblasts [MEFs] survive eight to 10 population doublings before they undergo premature cellular senescence. This senescence has been attributed to “culture shock” due to the nonphysiological conditions in which cells are grown [Sherr and DePinho 2000]. Accordingly, it has been recently observed that senescence of MEFs in vitro can be abrogated by maintaining cells in a more physiological oxygen environment (3% O2) [Parrinello et al. 2003]. The mechanism leading to this reduction in senescence appears to be tied to the decrease in DNA damage that the cells endure in hypoxic tissue culture conditions that are termed “normoxia” [i.e., atmospheric O2 levels, 21%]. A similar result has also been observed for oncogene-induced senescence, in which oncogenes such as Ras can induce premature senescence of MEFs [Serrano et al. 1997; Lee et al. 1999]. When grown in the presence of antioxidants or in lower oxygen tensions, however, Ras-expressing MEFs continue to proliferate [Lee et al. 1999]. These data suggest that reduction in oxidative damage due to reactive oxygen species (ROS) is sufficient to inhibit premature senescence.

Normal tissues are not typically exposed to the high levels of oxygen found in the environment. In vivo oxygen levels range from roughly 2%–3% in the brain, liver, and myocardium; 9%–10% in the spleen; and up to 13%–14% in the alveoli of the lung (Vaupel et al. 1989). Lower oxygen levels characterize normal and pathologic states including wound healing, ischemic disease, and cancer. The hypoxia-inducible factors (HIFs) are a family of transcriptional regulators that are important in the cellular response to hypoxia. They transcriptionally control a diverse number of genes including those involved in glycolytic metabolism, vascular remodeling, and erythropoiesis. HIFs are regulated primarily at the level of protein stability by the von Hippel Lindau protein [VHL], which directs the HIFα subunits to the proteosome for rapid degradation in oxic conditions [Kim and Kaelin 2004; Schofield and Ratcliffe 2004]. In the mildly hypoxic conditions common to many tissues, HIFα subunits are stabilized and active [Stewart et al. 1982; Bedogni et al. 2005]. Active HIF therefore correlates with resistance to premature senescence, both of which occur in physiological oxygen levels.

Whether HIFs play a direct role in preventing senescence under hypoxic conditions has not been determined. Recently, it has been observed in some endometrial cancer cell lines that modulation of the HIF pathway can affect senescence. Overexpression of a key negative regulator of HIFs [EGLN1], or use of a HIF inhibitor [YC-1], brought about proliferative arrest and the onset of a senescence-like phenotype [Kato et al. 2006]. Collectively, these observations prompted us to ask whether HIFs were able to prevent senescence in a ge-
RESULTS AND DISCUSSION

Loss of HIF1α causes premature senescence in MEFs in aerobic conditions

In order to determine if HIF1α contributes to the prolonged life span of MEFs grown under low oxygen conditions (Parrinello et al. 2003), we derived MEFs from a conditional loxP HIF1α MEFS to test the hypothesis that HIF1 may act to delay premature senescence. We found that HIF1α does play a role in the enhanced proliferation of cells under low oxygen conditions, and modulates senescence in response to hypoxic conditions (21% O₂). This effect was also seen when oxidative stress was induced by γ-irradiation to MEFs maintained in physiological oxygen tensions. We further found that the anti-senescent effect of HIF1α is mediated in part by the transcriptional regulation of the macrophage migration inhibitory factor (MIF). Together, these findings offer new insights into the modulation of the tumor suppressor mechanism of senescence and its regulation under physiological conditions.

MIF is a HIF1α-dependent, hypoxia-inducible gene

In order to identify a potential mechanism for how HIF1α delays senescence, we considered previously identified hypoxia-induced genes. One potential candidate is the macrophage MIF, which has been shown to negatively regulate p53 and prolong the life span of presenescent MEFs (Hudson et al. 1999, Fingerle-Rowson et al. 2003). Because MEFs derived from HIF1α⁺/− animals can be immortalized with SV40 large T antigen, regulation of senescence by HIF1α likely occurs upstream of p53 and Rb (Ryan et al. 1998). As a regulator of p53, MIF would therefore seem to be a potential link between hypoxia and senescence.

MIF was first found to be hypoxia inducible in a head and neck cancer cell line and in a cervical carcinoma cell line (Koong et al. 2000), and later in glial tumor cells (Bacher et al. 2003). In the latter study, the MIF promoter was found to be hypoxia inducible, suggesting regulation of MIF at the transcriptional level. How the promoter is regulated by hypoxia, however, was undetermined.

We first sought to determine if hypoxic regulation of MIF was dependent on HIF1α by using HIF1α knockout MEFs cultured under normoxic and hypoxic conditions. As seen in Figure 2A, GLUT1 mRNA is highly induced in wild-type cells but not in HIF1α-deficient cells following 24 h of hypoxia. MIF expression demonstrated the same behavior, and hypoxia-induced MIF mRNA transcribed into increased MIF protein as well (Fig. 2B). Interestingly, excision of HIF1α resulted in decreased expression of MIF (and to a lesser extent GLUT1) even in normoxic conditions, confirming the potential role for HIF1α in normoxia. Thus, we conclude that MIF regulation occurs at the transcriptional level, and is HIF1α dependent.
MIF is necessary and sufficient to delay senescence of MEFs downstream from HIF1α

To determine if expression of MIF is required to delay senescence, we designed a retroviral short hairpin RNA (shRNA) construct to stably knockdown MIF expression. MEFs were infected with this construct or a GFP control, selected, and then tested for MIF expression. As seen in Figure 3, A and B, our shRNA construct effectively knocked down the expression of MIF in both normoxic and hypoxic cells in a stable manner, while another hypoxia-induced gene (GLUT1) was unaffected and remained hypoxia inducible.

These cells were then plated and counted as before in

Figure 3. MEFs downstream from HIF1α

In order to investigate how HIF1α regulates MIF transcription, we cloned a 1-kb fragment of the murine promoter that has been shown to be hypoxia inducible (Bacher et al. 2003) into pGL3basic, and performed deletion analyses and reporter assays. We found that HIF1α overexpression alone was sufficient to activate the MIF promoter in normoxia (Supplementary Fig. 2). Large deletions revealed that the hypoxia inducibility of the promoter was dependent on two regions: one between –54 and –26, and the other between the transcriptional start site and +20 (Fig. 2C; cf. constructs A and B, and cf. constructs B and F). These regions are denoted by arrows in Figure 2C. Based on promoter database analysis (http://www.genomatix.de), the former region contains a putative overlapping hypoxia response element (HRE) and cyclic-AMP response element (CRE), as well as an Sp1 site, while the latter contains a second putative HRE/CRE (Fig. 2D). Specific mutation of the HRE or the Sp1 sites alone in the upstream region did not ablate hypoxia responsiveness, but did reproducibly reduce hypoxic induction. Mutation of both sites together did, however, significantly reduce hypoxia responsiveness (p < 0.05) (Fig. 2E). These results suggest that the upstream HRE is not functional in the classical sense of a sequence specific promoter-binding site, or that HIF1α can be recruited either by direct DNA binding or through recruitment by a cofactor. In the latter case, it is notable that HIF1 and Sp1 have been found to cooperate in the induction of other hypoxia-inducible genes (Miki et al. 2004), and may be acting in this way on the MIF promoter. In the second region, mutation of the HRE/CRE completely abolished hypoxic induction (Fig. 2E), suggesting this site is a functional HRE. In accordance with this finding, Baugh et al. (2006) have recently reported a similar site in the human MIF promoter.

We next wanted to determine if HIF1α is capable of binding to the MIF promoter directly in vivo. To do so, we performed chromatin immunoprecipitation (ChIP) assays. Similar to the GLUT1 HRE, PCR amplification of DNA that coimmunoprecipitated with an anti-HIF1α antibody compared with an IgG control resulted in specific enrichment of the MIF HRE region, but not a region 1 kb upstream (“MIF control”) (Fig. 2F). These data suggest that HIF1α is capable of binding the hypoxia responsive region of the MIF promoter directly in a living cell.
a proliferation/senescence assay. As in the case of HIF1α, reduced expression of MIF caused a significant growth delay in hypoxia, and premature proliferative arrest in normoxia (Fig. 3C). This arrest correlated with increased SA-βgal staining [Fig. 3D]. In agreement with the growth curves, loss of MIF expression in normoxia caused a significant decrease in DNA synthesis, as determined by 3H-thymidine incorporation [Supplementary Fig. 3A]. In addition, we found that this effect is dependent on p53. p53+/− MEFs failed to demonstrate any deficiency in HIF1α target gene expression [Supplementary Fig. 3B–D]. Together, these data suggest that expression of MIF is critical to avert p53-dependent cellular senescence.

To determine if MIF expression is sufficient to rescue premature senescence due to loss of HIF1α, we next retrovirally transduced MIF into the conditional HIF1α knockout MEFs. Following selection, these cells were infected with either Ad-Cre or Ad-empty as before to excise HIF1α [Fig. 3E], and then plated in a senescence assay. As shown in Figure 3F, ectopic expression of MIF partially rescued the effect of loss of HIF1α in normoxia. These data suggest that MIF is necessary and sufficient to delay senescence downstream from HIF1α. The lack of complete rescue, however, suggests the MIF may not be the only HIF1α target gene involved in this response.

It is noteworthy that a role for MIF in regulating proliferation and senescence in MEFs has been previously suggested. MIF overexpression has been linked to a delay in senescence through a decrease in p53 activity in MEFs (Hudson et al. 1999). Additionally, embryonic fibroblasts have been isolated from MIF knockout mice. While these animals do not have any overt phenotype, MIF-deficient MEFs display premature growth arrest at lower saturation densities (i.e., lower number of cells at confluence) (Fingerle-Rowson et al. 2003). While these results agree with our data in principle, the phenotype we have observed is more severe. In our studies MEFs demonstrate decreased DNA synthesis and proliferation immediately upon loss of MIF [Supplementary Fig. 3A]. This difference may be attributable to a difference in chronic versus acute loss of MIF. It is possible that chronic loss of MIF results in a developmental compensation mechanism to deal with potentially higher p53 activity. This type of effect has been seen for the Rb family proteins, in which acute loss of Rb has a more severe effect than chronic loss owing to compensation by the Rb family member p107 (Sage et al. 2003). Thus, our studies, utilizing methodologies that result in acute loss of both HIF1α and MIF, demonstrate a functional link between hypoxia, HIF1α, and the regulation of senescence through MIF expression.

**Loss of HIF1α sensitizes MEFs to γ-irradiation under physiological oxygen concentrations**

Hypoxia and HIF1α are thought to play critical roles in the development of cancer. As solid tumors surpass a size of a few millimeters, they develop regions of severe hypoxia that lead to the optimal activation of HIF1α and its downstream target genes. HIF target genes are involved in crucial aspects of tumor development, including angiogenesis, metabolic adaptation, survival and metastasis. While there may be significant benefit to inhibiting HIF1α in tumors, our data suggest that there may also be significant effects in adjacent normal cells. As senescence in culture has been likened to aging in vivo (Campisi 2005), a reduction in HIF1α activity may lead to premature senescence and aging, particularly following therapeutic radiation exposure that results in severe oxidative stress.

To test the theory that loss of HIF1α activity would sensitize normal cells to radiation exposure under physiological oxygen concentrations (2% O2), we subjected the HIF1αfl/fl MEFs infected with Ad-Cre or Ad-empty adenoviruses to sublethal doses of γ-irradiation (0.5 Gy or 2 Gy). While we found that wild-type cells [infected with Ad-empty] were only moderately affected by these radiation doses, and did not display any arrest during the course of the assay, HIF1α-deficient cells demonstrated a substantial sensitization to radiation [Fig. 4A]. After 12 d of culture in 2% O2 following irradiation, both the 0.5 Gy- and 2 Gy-treated Cre-infected cells were more than eightfold fewer than their Ad-empty counterparts. Again, we confirmed that cell death was not occurring
by annexin/PI staining [Supplementary Fig. 1B]. Furthermore, the HIF1α-deficient cells demonstrated significant SA-βgal staining and assumed a senescent morphology [Fig. 4B]. Greater than 27% of the Cre-infected cells stained positive for SA-βgal following 0.5 Gy of radiation, and >32% following 2 Gy of exposure. In contrast, the Ad-empty-infected cells showed only 1.4% and 2.4% blue cells, respectively [Fig. 4C]. Thus, HIF1α loss sensitizes cells to radiation-induced senescence. Accordingly, we also found that knockdown of MIF simulates HIF1α in modulating senescence through the regulation of MIF. Error bars indicate standard deviation.

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