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Inactivation of the CYLD Deubiquitinase by HPV E6 Mediates Hypoxia-Induced NF-κB Activation

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SUMMARY

The biochemical mechanisms that underlie hypoxia-induced NF-κB activity have remained largely undefined. Here, we find that prolonged hypoxia-induced NF-κB activation is restricted to cancer cell lines infected with high-risk human papillomavirus (HPV) serotypes. The HPV-encoded E6 protein is necessary and sufficient for prolonged hypoxia-induced NF-κB activation in these systems. The molecular target of E6 in the NF-κB pathway is the CYLD lysine 63 (K63) deubiquitinase, a negative regulator of the NF-κB pathway. Specifically, hypoxia stimulates E6-mediated ubiquitination and proteasomal degradation of CYLD. Given the established role of NF-κB in human carcinogenesis, these findings provide a potential molecular/viral link between hypoxia and the adverse clinical outcomes observed in HPV-associated malignancies.

INTRODUCTION

It has been over 50 years since the seminal observation by Thomlinson and Gray that intratumoral hypoxia is associated with resistance to radiation therapy (Thomlinson and Gray, 1955). In the ensuing decades, mounting clinical and experimental evidence has established the influence of hypoxia on tumor biology. For example, the identification of intratumoral hypoxia in patients with cervical and head and neck cancer is associated with an increased risk for local recurrence after radiation, the presence of lymphatic and hematogenous metastases, and reduced overall survival (Tatum et al., 2006). Moreover, hypoxia is associated with resistance to not only radiation therapy but also cytotoxic chemotherapy (Harris, 2002; Le et al., 2004; Subarsky and Hill, 2003). Additional investigations have further extended the importance of hypoxia in the malignant progression of other tumor models, such as sarcomas, breast cancer, and prostate cancer (Tatum et al., 2006; Vaupel et al., 2001, 2002).

As a solid tumor grows, hypoxia invariably occurs as a consequence of aberrant neoangiogenesis, cancer-associated anemia that results in reduced oxygen-carrying capacity of blood, increased oxygen demand of the growing tumor, and abnormal oxygen diffusion due to imbalances in directional microcirculation (Hockel and Vaupel, 2001). In addition to reduced oxygen tension, the hypoxic environment is also characterized by acidosis and diminished micronutrient availability. Thus, during carcinogenesis, premalignant and malignant cells must adapt to the harsh hypoxic microenvironment and do so through both genomic and nongenomic mechanisms (Hockel and Vaupel, 2001). Indeed, hypoxia results in genomic instability manifested by increased rates of gene amplification, point mutation, and chromosomal rearrangement that add to the genomic complexity of tumor cells (Hockel and Vaupel, 2001; Reynolds et al., 1996; Young et al., 1988). Through selection pressures, this genomic instability leads to outgrowth of clones that manifest survival and even proliferation advantages as well as resistance to antineoplastic therapy.

SIGNIFICANCE

Intratumoral hypoxia is a critical factor in the poor clinical outcomes of human malignancies, most notably cervical and head and neck cancers. Here, we find that prolonged hypoxia-induced NF-κB activation is not a generalized phenomenon among cancer cells but rather is restricted to human papillomavirus (HPV)-positive cancers, such as cervical and head and neck cancers. Under hypoxic conditions, the HPV-encoded E6 protein inactivates the CYLD tumor suppressor, a negative regulator of the NF-κB pathway, and thereby allows for unrestricted activation of NF-κB. Because NF-κB-induced genes promote survival, proliferation, and angiogenesis, our findings illustrate how a common human virus adapts to hypoxia and helps account for the aggressive tumor biology associated with hypoxia.

Nongenomic cellular adaptations to hypoxia have been more thoroughly studied and include upregulation of angiogenesis, oxygen transport, glycolysis, and glucose uptake (Harris, 2002). Many of these adaptations are mediated by the transcription factor hypoxia-inducible factor alpha (HIFα), which drives expression of hypoxia-response genes, such as vascular endothelial growth factor (*VEGF*). In addition to HIFα, other transcription factors have been reported to be activated by hypoxia. For example, hypoxia-induced activation of the NF-κB family of transcription factors has been observed in some tumor models (Koong et al., 1994; Royds et al., 1998). NF-κB is a family of transcription factors that induce a transcriptional response that results in the expression of proteins that promote survival, proliferation, angiogenesis, invasion, and metastasis (Baldwin, 2001). Accordingly, hypoxia-induced NF-κB activation represents another potential mechanism whereby tumor cells could adapt to the inhospitable hypoxic milieu.

Whereas constitutive NF-κB activity has been implicated in the malignant progression of numerous hematologic and solid malignancies (Basseres and Baldwin, 2006), a comprehensive analysis of the timing and duration of hypoxia-induced NF-κB activation has not been performed to our knowledge. Moreover, the biochemical mechanisms that underlie hypoxia-induced NF-κB activity have remained largely undefined. In the current study, we observed that prolonged hypoxia-induced NF-κB activation was restricted to human papillomavirus (HPV)-positive cancer cells and was mediated by an effect of the HPV-encoded E6 protein on polyubiquitination and subsequent degradation of the CYLD lysine 63 (K63) deubiquitinase.

RESULTS

Prolonged Hypoxia-Induced NF-κB Activation Is Restricted to HPV-Infected Cell Types

Although hypoxia-induced NF-κB activation has been reported in various cell systems, a thorough analysis of the timing and extent of hypoxia-induced NF-κB activation across a wide range of malignant cell types has not been performed. We performed electrophoretic mobility shift assays (EMSAs) to screen 32 human cancer cell lines of epithelial or mesenchymal origin for hypoxia-induced (1% O_2) NF-κB activation. Only 4 of 32 cell lines exhibited hypoxia-induced NF-κB activation at 24 or 48 hr time points, and all four of these cell lines represented squamous cell carcinomas of the cervix (HeLa, SiHa, and Me180; n = 3) or head and neck (HEp2; n = 1), all of which are infected with high-risk HPV serotypes (Figure 1A; see also Table S1 available online). Strikingly, in HPV-negative cervical (HT3 and C33A) and head and neck (CAL27) cancer cell lines, hypoxia resulted in either no change or a decrease in NF-κB activity (Figure 1A; Table S1). These results were confirmed by transient transfection of an NF-κB-driven reporter (Figure 1B; Table S1). Electrophoretic mobility supershift analyses revealed that the NF-κB complexes were composed of p65 and p50, components of the classical NF-κB pathway (Figure 1C). Of note, the p65 antibody employed for this assay did not retard the migration of the band representing p65-p50 heterodimers but rather prevented p65-p50 heterodimers from binding to the radiolabeled NF-κB oligonucleotide probe and therefore resulted in a reduced signal attributable to p65-p50 DNA binding (Lee and Ziegler, 2007).

We evaluated HPV-positive and -negative tumor xenografts for hypoxia-induced NF-κB activation. HeLa (HPV18-positive squamous cell carcinoma of the cervix) and CAL27 (HPV-negative squamous cell carcinoma of the head and neck) subcutaneous xenografts were harvested from the flanks of nude mice 1 hr after intraperitoneal injection of the hypoxia marker pimonidazole. In HeLa xenografts, regions of hypoxia as detected by pimonidazole staining were associated with nuclear p65 staining, whereas relatively nonhypoxic regions manifested predominantly cytoplasmic p65 expression (Figure S1A). We did not observe any nonspecific staining by a control antibody of the same isotype (rabbit IgG) as the p65 antibody (data not shown). In contrast, p65 nuclear staining was actually reduced in hypoxic regions in CAL27 xenografts (Figure S1B), a result concordant with the diminished NF-κB activation observed in CAL27 cells in vitro (Figure 1A).

A detailed time-course analysis of HPV-positive HeLa and SiHa (HPV serotype 16) cells revealed that hypoxia-induced NF-κB activation occurred rapidly (i.e., within 15 min) and was sustained for at least 48 hr (Figure 1D, top panels). Hypoxia did not affect the electrophoretic mobility of AP1 or Oct-1 complexes (Figures 1D and 1E), indicating that hypoxia-induced transcription factor activation is not a generalized phenomenon. When hypoxic HPV-positive cells were returned to normoxia (21% O_2), NF-κB levels gradually declined (Figure 1E). Specifically, NF-κB activity was maintained for several hours prior to a return to the baseline level of NF-κB activity exhibited at normoxia prior to initial exposure to hypoxic conditions. Some HPV-negative cell lines exhibited a modest degree of transient hypoxia-induced NF-κB activation that abated after 3 hr (Figure 1F). For example, DLD-1 cells, an HPV-negative colon cancer cell line, exhibited activation of NF-κB after 1 and 3 hr of hypoxia exposure, but at later time points, cells no longer demonstrated heightened NF-κB activity. Taken together, these data indicate that prolonged hypoxia-induced NF-κB activation is restricted to HPV-positive cancer cell models.

Activation of NF-κB is typically mediated by biochemical signaling events that converge on the IκB kinase (IKK) complex, which consists of the IKKγ (NEMO), IKKα, and IKKβ isoforms (Karin, 2006; Chen, 2005). Whereas IKKγ is the essential regulatory unit of the IKK complex, IKKα and IKKβ are catalytic subunits that are operative in the alternative and classical NF-κB pathways, respectively, and mediate NF-κB activation by phosphorylating IκB inhibitory proteins, thereby targeting IκB for ubiquitination and subsequent proteasomal degradation. Because our electrophoretic mobility supershift experiments indicated that hypoxia-induced NF-κB activation involved members of the classical NF-κB pathway, we evaluated the effects of hypoxia on IKKβ activity. Prolonged hypoxia (24 hr) induced IKKβ kinase activity in HeLa and SiHa cells (Figure S2A). We also investigated the timing of IKKβ activation in HPV-positive and -negative cell models. In HPV-positive cells (HeLa and SiHa), hypoxia-induced IKKβ activation occurred rapidly and was sustained (Figure S2B, top panels). In contrast, in HPV-negative cells, hypoxia either failed to induce IKKβ altogether (C33A) or resulted in transient IKKβ activation at 1 hr that returned to baseline levels after 4 hr (HT3 and DLD-1 colon cancer cells; Figure S2B, bottom panels). The timing of IKKβ activation correlated with the timing of NF-κB activation observed in the gel shift assays (Figures 1D and 1F)