UNIVERSITY OF CALIFORNIA, SAN DIEGO

Hypoxic Regulation of the NKG2D Ligand, H60

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by

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ABSTRACT OF THE THESIS

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Hypoxia in the context of cancer has been well studied as it has been shown that tumors that are in hypoxic conditions tend to become malignant or metastatic. There is evidence that hypoxia is able to modulate tumor immunogenicity, however this phenomenon has not been well characterized. Here, we look at the effects of hypoxia on tumor immunogenicity from the perspective of NK cell recognition. We find that hypoxia decreases the expression of the NKG2D ligand, H60 post-transcriptionally but not post-translationally, and that this down-modulation of the ligand prevents tumor recognition by NK cells.

Introduction

Previously, we showed that IFN down-regulates H60 by STAT1 via change in transcript. We are therefore interested in identifying new stimuli can down-regulate the NKG2D ligands. In the present study, we show that the NKG2D ligand H60 is reduced in the presence of hypoxia, and that this reduction prevents NK cells from recognizing the target. Our results show that hypoxia can down-regulate H60 to a similar extent as IFN but through a distinct mechanism.

Chapter 1: General Introduction.

1.1 Role of Hypoxia in Cancer

Hypoxia in the context of cancer has been well studied as it has been shown that tumors that are in hypoxic conditions tend to become malignant or metastatic.[1, 3] This is thought to occur by the upregulation of gene programs involved in cell survival such as angiogenesis, anaerobic metabolism, and radiation resistance.[11, 12] While hypoxia is known to upregulate many transcription factors, the effects of hypoxia are generally studied through the Hypoxia Inducible Factors (HIF) . HIF is a heterodimeric complex, which activates genes involved in homeostasis. This dimer contains an oxygen insensitive β-subunit (HIF1β) and one of three oxygen sensitive α-subunits (HIF-1α, HIF2α, or HIF3α).[22] While HIF1β is normally present in the cytoplasm under oxygenated conditions, the a-subunit is rapidly degraded by the E3 ligase, Von-hippel-lindau (VHL).[21] The importance of the HIF transcription factors in tumor progressions has been assessed in the HIF-1α knockout mice and HIF2a knockdown mice, in which tumor growth is impaired.[16, 23] Because survival traits are generally induced by hypoxia, it is also possible that exposure to low levels of oxygen leads to a response that allow tumor cells to escape from immune surveillance. This effect has already been established in the human system with the NKG2D ligand MICA, as it was found that tumors which have been incubated under hypoxic conditions cleave MICA off the cell surface. [18] The mouse NKG2D ligands, however, have not been investigated for this property.

1.2 The Regulation of NKG2D Ligands

The mouse NKG2D ligands, histocompatibility 60 (H60), retinoic acid inducible (RAE), and MULT1, play important roles in tumor surveillance by natural killer (NK) cells. While these ligands are usually absent from normal tissues, they can be induced upon cellular stresses such as carcinogenesis and viral infection, and are expressed in a broad range of carcinomas and some hematopoietic malignancies.[6] In mice, the interaction of cell surface MIC molecules with the C-type lectin-like NKG2D receptor on NK and effector T cells leads to the activation of innate and adaptive immune responses with the subsequent lysis of the tumor cells. Thus, it has been proposed that H60-NKG2D interactions are critical to the immune surveillance function of NK and perhaps NKT cells.[7] There is evidence that the shedding of MIC ligands in a soluble form represents a mechanism of tumor cell escape from NKG2D-mediated immune surveillance.[18, 21] Overexpression of the NKG2D ligands H60 and RAE on tumors has been shown to be sufficient in causing cytotoxic lymphocyte (CTL)-independent tumor rejection.[7] The final piece of evidence that these interactions are important in tumor surveillance is that the NKG2D knockout mouse ($klrk^{-/-}$) has a higher proportion of prostate cancer incidence than wildtype TRAMP mice.[8]

Chapter 2: Hypoxic regulation of the NKG2D Ligand, H60

2.1 Hypoxia lowers surface expression of H60

To test whether hypoxia reduced surface levels of NKG2D ligands on tumors, we cultured mouse fibrosarcomas under normoxic and hypoxic conditions as described above and measured their surface levels of H60, RAE and MULT1. We found that treatment with hypoxia reduced surface levels of H60 while not changing the other NKG2D ligands significantly. (Fig 1a and data not shown) In all experiments, tumors with endogenous H60 had a reduction ranging from 20% to 80%. To test whether this effect was due to post-translational regulation, as described for MICA (a human counterpart to H60), we took cell lines that had transduced H60 and cultured them in hypoxia. This effect was specific for endogenous H60, since cells with transduced H60 did not decrease H60 expression while cells expressing endogenous H60 did.

Hypoxic signals are relayed in part through HIF-1α. This can be activated by $CoCl_2$ as Co^{2+} is able to bind to VHL, inhibiting its ubiquitin E3 ligase activity. To determine if the regulation of H60 by hypoxia occurred through HIF-1α, we cultured MCA sarcomas with and without cobalt chloride and measured their H60 expression. Similar to before, H60 surface levels were decreased in tumors treated with 150 uM $CoCl_2$ in comparison to untreated tumors. (Fig 1b-c). Similar to the previous experiment, ectopically expressed H60 was not reduced by cobalt chloride treatment. Previously, we have shown that IFN can down-regulate H60. Therefore, to compare the relative down-regulation of IFNg to the lowered surface expression seen by HIF induction, we cultured

tumors with IFNg with and without $CoCl_2$. We observed that IFNg down-regulation was consistently lower than the reduction of H60 surface expression by $CoCl_2$. (Fig. 1D) These data indicate that surface H60 is being reduced in the presence of hypoxia, and that HIF-1α activity is sufficient to exert this effect.

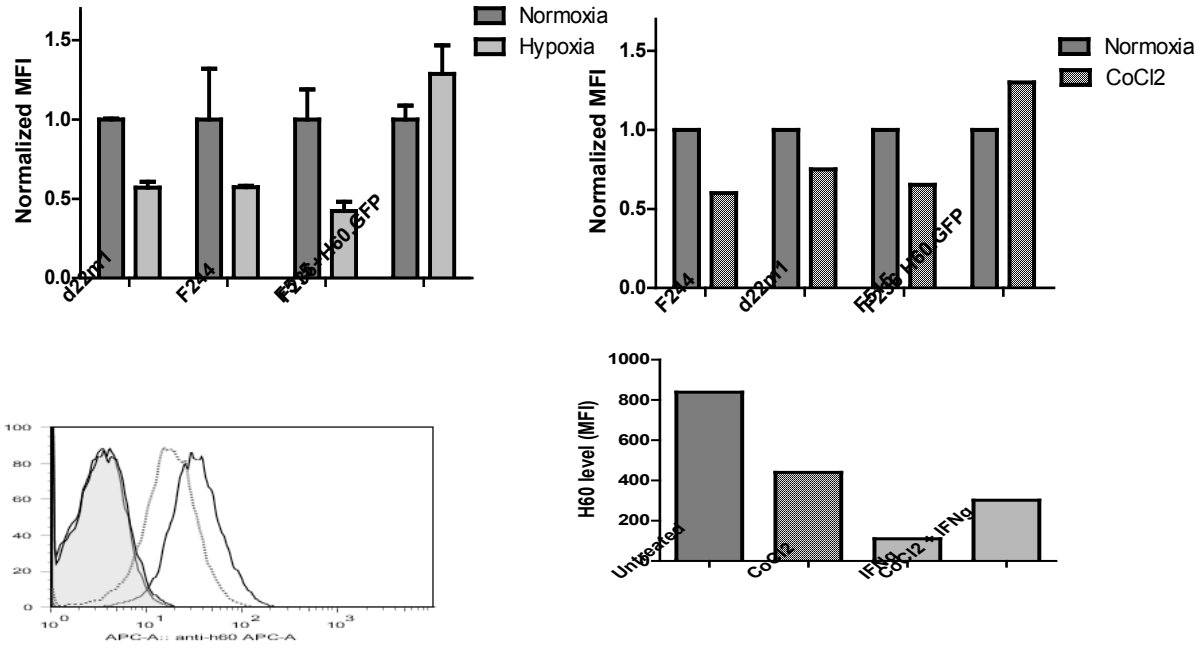

Figure 1. Hypoxia lowers surface expression of H60 (A) The MCA sarcomas d22m1, f244, and f515 were cultured in hypoxia (1% O_2) for 24 hours and in normoxia (20% O_2) for 24 hours. Data are normalized to normoxic controls within cell lines and show a reduction of surface level H60. (B) Various cell lines were treated with and show a reduction of surface level H60. Only F236 with transfected H60 was shown to increase H60. (C) Representative plot of H60 expression with cobalt chloride treated tumors (dotted line) and untreated tumors (Black solid line). Shaded histograms (overlapping) represent the isotype controls. (D) d22m1 was cultured with cobalt chloride, IFNγ, and cobalt chloride with IFNγ

2.2 Hypoxia does not lower H60 transcript levels.

To better understand the regulation of H60, we cultured fibrosarcomas under normoxic and hypoxic conditions and measured the mRNA levels of H60. Surprisingly, there was no decrease in H60 mRNA in the hypoxic conditions; rather mRNA was seen

to increase slightly but not significantly. (Fig 2a) This was again validated by culturing tumors with cobalt chloride, and measuring the H60 mRNA. (Fig 2b) These data indicate that reduction of H60 by hypoxia occurs at the post-transcriptional level; however taken with the data from figure 1, the regulation is also not posttranslational.

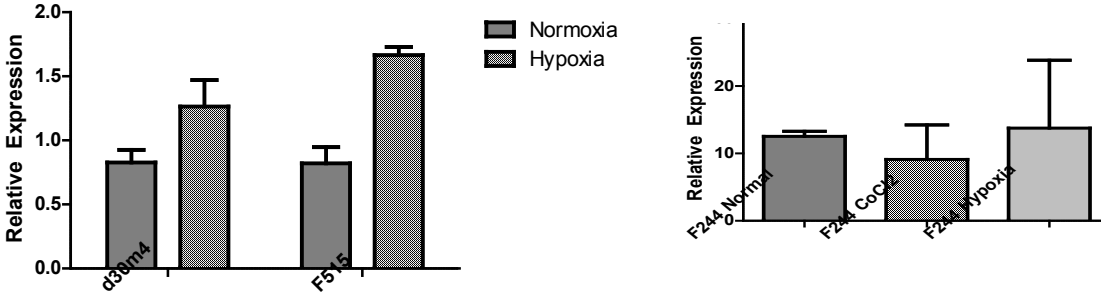

Figure 2. H60 mRNA does not change with exposure to hypoxia. The MCA sarcoma cell lines d30m4, F515, and F244 mRNA transcripts of H60 were quantified by quantitative PCR from total RNA isolated from cells cultured for 24 hours under normoxic or hypoxic (A) or untreated and $CoCl_2$ conditions (B). Shown are SEM. All results were reproduced at least twice for each cell line.

2.3 miRNAs induced by hypoxia potentially regulate H60

There have been numerous reports of miRNAs being induced by hypoxia.[6] To see if H60 could potentially be regulated by a miRNA that was induced by hypoxia, we examined the 3` untranslated region (UTR) of H60, checking for any binding sites to known miRNAs that were upregulated by hypoxia, we found that while mir-210, the most commonly identified miRNA associated with hypoxia, did not have any binding sites on the H60 3' UTR, mir-30b had two sites and mir-181b had four sites. (Fig 3) Probabilistic analysis was performed to assess the probability of finding the number of binding sites as were observed in the 3' UTR, finding that there was a 2% probability that four mir-181 binding sites would be present in the H60 3' UTR and that there was a 30%

probability that two mir-30b was found. This indicates a possible mechanism that hypoxia might be working through to decrease expression of H60.

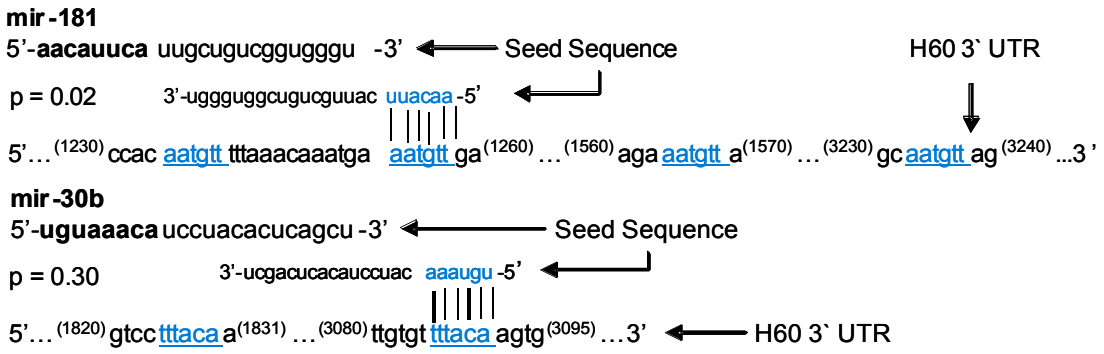

Figure 3. Hypoxia regulated miRNA have binding sites in the H60 3` UTR . p-values represent the probabilities of having as many or more miRNA binding sites in the 3' UTR randomly.

2.4 Cobalt Chloride reduces NK cell lysis of tumors

To test the physiological role of the hypoxic regulation of H60, we tested whether HIF induction resulted in reduced NK cell recognition of tumors. We found that tumors treated with cobalt chloride were not as well recognized as untreated tumors. (Fig 4) Although killing did not exceed 10% , cobalt chloride tumors consistently had less killing at all ratios. To test whether this killing was perforin dependent, the kill assay was performed in the presence of EGTA . We found that there was no significant killing, with killing never exceeding 3%, suggesting that receptors such as TRAIL are not mediating the killing (data not shown). This suggests that HIF induction decreases the recognition of tumors by NK cells.

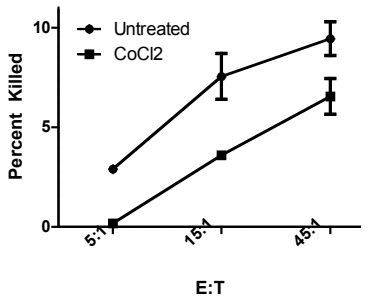

Figure 4. Cobalt chloride treatment inhibits NK cell mediated killing. The tumor cell line F244 was treated with medium or 150 uM cobalt chloride and used as targets in a kill assay against IL-2 activated NK cells.

2.5 Discussion

Hypoxia is known to change the internal physiology of the tumor in many ways, but the effect of hypoxia on tumor immunogenicity is not well studied. Here, we show that hypoxic conditions cause the reduction of the NKG2D ligand H60 on tumors and that this reduction likely occurs through HIF activation. We go on to show that this reduction occurs at the post-transcriptional but not post-translational level, and we identified some possible miRNAs that could be responsible for the observed reduction. Our results also suggest that hypoxia decreases the immunogenicity of the tumor from the perspective of NK cells.

This paradigm is strengthened by the published results that hypoxia incubated tumors are less immunogenic from the perspective of CTLs, despite it being discovered in the human system. While this group did not identify a specific downstream protein that directly prevents tumors from being recognized by CTLs, the finding does support that HIF induction causes a reduction in generalized tumor recognition. Despite these findings, the data is still nebulous due to the increasing evidence that hypoxia induces

resistance to apoptosis, the mechanism by which CTLs kill tumor targets.[14] NK cells are thought to release perforin which disrupts the membrane; however it is also believed that they can kill by inducting apoptosis as well. Pro-apoptotic protein families such as Bcl-2 have been implicated in this resistance, however, further research needs to be done to see if this induction is cell type specific. When performing the kill assays, cobalt chloride treated tumor cells had higher basal death than untreated tumor cells.

Interestingly, it has been shown that hypoxia can induce a proinflammatory environment for immune cells. This also fits in with this study, as inflammation has been shown to cause tumor progression in many model systems.[3] While inflammatory environments by nature may not be hypoxic, other stimuli reactive oxygen species and NO can cause HIF accumulation in the cytoplasm, inducing a hypoxia gene program.[4] This phenomena has also been observed in dendritic cells, specifically that they produce more proinflammatory cytokines, while preventing MHC class II expression.[13] This effect of inflammation to not only induce a hypoxic gene program in immune cells to prevent antigen recognition, but also to help tumors activate HIF-1α genes could play a role in cancer progression.

Further studies will to be performed to identify the exact mechanism of how H60 is reduced off the cell surface along with the characterization of other NK signals that could be mediating this reduced recognition.

Chapter 3: Materials and Methods

3.1 Cell Culture and Reagents

Mouse MCA sarcoma tumor cell lines were generated from mice treated with the carcinogen MCA as described.[2] Some cell lines were transduced to express H60.[2] Cells were cultured in RPMI/10% fetal bovine serum with penicillin/streptomycin. Hypoxia culture conditions was defines as 1% O_2, 5% CO_2, while normoxia culture conditions were defined as 20% O_2 and 5% CO_2.

3.2 Flow Cytometry

Cells were harvested using 2.5 mM EDTA without trypsin and stained with antibodies to H60, RAE, MULT1 (R&D Systems), or appropriate isotype controls (eBioscience). Secondary Abs used were goat anti-rat IgG-APC (eBioscience).

3.3 Quantitative RT-PCR

cDNA synthesis kits were purchased through Applied Biosystems. H60, 18S rRNA, RAE and MULT1 primers were purchased from ValuGene. The sequences of the primers are listed previously.[2] The sequence of the 18S rRNA primers is 18S RNA: sense: GATTAAGTCCCTGCCCTTTGTACA antisense: GATCCGAGGGCCTCACTAAAC probe: VIC-CGCCCGTCGCTACTACCGATTGG. Total RNA was extracted using Trizol (Invitrogen, Friendswood, Texas). RT reactions were normalized to 18S rRNA for all transcripts.

3.4 NK Cell Killing Assay

NK cells were isolated from $RAG2^{-/-}$ 129/Sv mice and cultured for 7 days in the presence of 1000 u/mL IL-2. They were then cocultured with tumors at varying ratios for 8 hours,

after which they were stained with 7AAD as a marker for dead cells. All killing assays were performed at normoxia.

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