Hypoxia-induced Nucleophosmin Protects Cell Death through Inhibition of p53*

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Nucleophosmin (NPM) is a multifunctional protein that is overexpressed in actively proliferating cells and cancer cells. Here we report that this proliferation-promoting protein is strongly induced in response to hypoxia in human normal and cancer cells. Up-regulation of NPM is hypoxia-inducible factor-1 (HIF-1)-dependent. The NPM promoter encodes a functional HIF-1-responsive element that can be activated by hypoxia or forced expression of HIF-1α. Suppression of NPM expression by small interfering RNA targeting NPM increases hypoxia-induced apoptosis, whereas overexpression of NPM protects against hypoxic cell death of wild-type but not p53-null cells. Moreover, NPM inhibits hypoxia-induced p53 phosphorylation at Ser-15 and interacts with p53 in hypoxic cells. Thus, this study not only demonstrates hypoxia regulation of a proliferation-promoting protein but also suggests that hypoxia-driven cancer progression may require increased expression of NPM to suppress p53 activation and maintain cell survival.

Hypoxia is a physiological stress that can activate the cell death program and thus select for cells resistant to hypoxia-induced apoptosis. Indeed, many tumors can grow in hypoxic microenvironments, which is often associated with poor prognosis and less response to cancer therapy (1). While most tumor cells retain the ability to undergo apoptosis in response to hypoxic stress, they can become adaptive to hypoxia by increasing synthesis of factors that promote cell survival and proliferation (2). The transcriptional factor hypoxia-inducible factor-1α (HIF-1α) is a central mediator of hypoxic response (3). Under normoxic conditions, Hif-1α is rapidly degraded by the proteasome after being targeted for ubiquitination, a process that is dependent on the tumor suppressor von Hippel-Lindau protein (4). However, Hif-1α is stabilized under hypoxic conditions, which in turn transactivates a variety of genes in the adaptive response (3). Hif-1α has been shown to play essential roles in hypoxia-mediated apoptosis, cell proliferation, and tumor angiogenesis (5).

Nucleophosmin (NPM) is a multifunctional protein initially characterized as a nucleolar protein functioning in the processing and transport of ribosomal RNA (6). NPM is found to be more abundant in tumor and growing cells than in normal resting cells (6–15). In fact, NPM has been proposed as a tumor marker for human colon (9), ovarian (10), prostate (11), and gastric (12) cancers because NPM expression is markedly higher in these tumor cells than in the corresponding normal cells. NPM is also identified as a major gene product required for stem cell development (stemcell.princeton.edu). Conversely, NPM expression is down-regulated in cells undergoing differentiation or apoptosis. For example, NPM mRNA is decreased in HT29-D4 colon carcinoma cells treated in vitro to undergo differentiation (13). The levels of NPM were significantly lower in the WEHI-231 B lymphoma cells and the human T cell leukemia Jurkat cells treated to undergo growth rest or apoptosis, as compared with untreated cells (14, 15). Similarly, repression of NPM expression by antisense strategy potentiates drug-induced apoptosis in the human HL60 leukemia cells (16). NPM appears to be the target for certain transforming oncogenes. Indeed, Zeller et al. (17) used DNA microarray technology for gene expression analysis to examine target sequences in human genome by the oncogenic transcription factor c-Myc and identified NPM as a Myc-responsive gene. NPM expression was 3.5-fold higher in myc-overexpressing avian bursal neoplasia than in normal bursa (18). NPM is also frequently found in the chromosomal translocation associated with several hematopoietic malignancies, such as acute promyelocytic leukemia (19), anaplastic large cell lymphomas (20), and myelodysplasia/acute myeloid leukemia (21).

We have characterized the response of NPM to hypoxia in human normal and cancer cells and demonstrated that NPM is strongly induced in response to hypoxia and protects cell death likely through inhibition of p53 activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—Normal lymphoblasts derived from a healthy donor were maintained in RPMI medium 1640 (Invitrogen) supplemented with 15% fetal bovine serum (FCS). Human embryonic kidney 293 cells (HEK293) and human normal fibroblasts derived from a healthy donor were grown in Dulbecco’s modified Eagle’s medium with 10% FCS and α-minimal essential medium with 20% FCS, respectively. The human cancer cell lines used in this investigation were: HCT116 (colon cancer), MCF-7 (breast cancer), PC-3 (prostate cancer), K562 (chronic myelogenous leukemia), and HL60 (promyelocytic leukemia). These cells were maintained in various media in accordance to the requirements. Two sets of cells approaching confluence were incubated in parallel at 37 °C in normoxia (humidified air with 5% CO2) or hypoxia within a modular incubator chamber (BioSpherix, Redfield, NY) filled with 0.1% O2, 5% CO2, and balance N2.

Immunoprecipitation and Immunoblotting—Whole cell extracts (5

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Hypoxia Induces NPM Expression

Because high levels of NPM expression have been found in a variety of human cancers (7, 9–15) and because many tumors contain hypoxic microenvironments (1), we wished to determine whether the NPM protein was elevated in hypoxic cancer cells. Five human cancer cell lines, HCT116 (colon cancer), MCF-7 (breast cancer), PC-3 (prostate cancer), K562 (chronic myelogenous leukemia), and HL60 (promyelocytic leukemia), were subjected to normoxia or hypoxia for 12 h before lysis. As shown in Fig. 1B, NPM expression was significantly increased in all tested cancer cell lines exposed to hypoxic conditions compared with cells incubated in normoxic conditions. Given that most cancer cells retain the ability to undergo hypoxia-induced cell death (25), elevated NPM expression in response to hypoxia by leukemia and other cancers raises the expectation that tumor cells may require increased expression of NPM to maintain cell survival under hypoxic conditions, and targeting this molecule may prove useful for cancer prevention and treatments.

RESULTS AND DISCUSSION

NPM Protein Is Induced by Hypoxia—Expression of NPM is induced by stresses like DNA-damaging UV irradiation (23) and oncogenic insults (17, 18). We thus examined NPM expression in hypoxic normal human lymphoblast and fibroblast cells. We found that the level of NPM protein was increased significantly in response to hypoxia after 6 h and last over a 24-h period (Fig. 1A, top panel). This coincides with hypoxia induction of HIF-1α (middle panel). NPM, as well as HIF-1α, was also induced in cells treated with CoCl2, a chemical mimic of hypoxia (Fig. 1A, lanes 5 and 10; Ref. 24).

FIG. 1. NPM protein is induced by hypoxia. A, human lymphoblasts or fibroblasts were incubated under normoxic (20% O2) or hypoxic (0.1% O2) or in the presence of 100 μM CoCl2, 100 μg of the indicated whole cell extracts were examined by immunoblotting, using antibodies specific for NPM (top), HIF-1α (middle), or β-actin (bottom). B, whole cell extracts (50 μg of total proteins) from human cancer cell lines HCT116, MCF-7, PC-3, K562, and HL60 exposed to hypoxia for 12 h were subjected to immunoblotting analysis with anti-NPM (top) or anti-β-actin (bottom).
NPM Protects against Hypoxic Cell Death of WT but Not p53-null Cells—
To assess the physiological role of the inductive response of NPM to hypoxia, we analyzed hypoxia-induced apoptosis in the presence of NPM overexpression and a reduced level of intracellular NPM. We first applied RNA interference to down-regulate the expression in human breast cancer MCF-7 cells and determined the effect on hypoxia-mediated apoptosis. After 24 h of co-transfection, luciferase activities were determined. The values represent the average luciferase activity of three independent experiments; bars indicate standard error.

NPM Protects against Hypoxic Cell Death of WT but Not p53-null Cells—
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Fig. 2. Hypoxia-induced transcriptional up-regulation of NPM. A, first strand cDNA was synthesized from the total RNAs extracted from normal lymphoblasts incubated under hypoxic conditions and subjected to RT-PCR analysis. Shown is a RT-PCR result obtained with 30 cycles of amplification of cDNA encoding NPM, HIF-1α, VEGF, and 28S rRNA, respectively. B, the promoter sequence of NPM from HEK293 cells along with pcDNA3 (vector) or pcDNA3-HIF-1α, and the transfectants were incubated under normoxic or hypoxic conditions. After 24 h of incubation, luciferase activities were determined. The values represent the average luciferase activity of three independent experiments; bars indicate standard error.

Fig. 3. NPM protects cells from hypoxia-induced cell death in WT and p53-null cells. A, specific inhibition of NPM expression by NPM-siRNA. MCF-7 cells were treated with NPM-siRNA or control siRNA oligonucleotides for 6 h under normoxic conditions and then subjected to hypoxia for 16 h. Whole cell extracts (100 μg of total proteins) were subjected to Western blot analysis with anti-NPM (top panel) or anti-β-actin (bottom panel). B, apoptosis in cells described in A was analyzed for caspase 3 activation by flow cytometry. The mean ± S.D. of three independent experiments is shown. C, overexpression of WT and mutant NPM proteins in WT and p53-null mouse embryonic fibroblasts cultured in normoxic conditions or hypoxia for 16 h. Western blot analysis was performed with anti-FLAG (M2). D, overexpression of NPM reduces hypoxic death of WT but not p53-null cells. Effect of NPM overexpression on hypoxia-induced cell death was determined after 3 days in 0.1% O₂ by trypan blue staining. Data represent the mean ± S.D. of three independent experiments.
tions (Fig. 3B). However, a significant increase of caspase-3 activity (2–3-fold) was observed in hypoxic cells expressing the NPM-siRNA, as compared with the control or mock-transfected hypoxic cells (Fig. 3B).

We next tested whether NPM overexpression could protect cell death induced by hypoxia. Because NPM has been shown to interact with the tumor suppressor p53 (22), we wished to determine whether the NPM protection against hypoxia-induced cell death involves p53. The genetically matched p53 WT and null mouse embryonic fibroblasts were transfected with vectors expressing WT NPM or a mutant variant with a deletion of the C-terminal 120 amino acids of NPM (NPMΔC) that is required for binding to p53 (22). High levels of FLAG-tagged NPM or NPM mutant proteins were achieved in the transfectants (Fig. 3C), whereas no FLAG-tagged proteins were detected in control vector samples (Fig. 3C, lanes 1, 4, 7, and 10).

We then tested the transfectants for sensitivity to hypoxia. Overexpression of WT NPM in p53 WT cells increased cell survival in hypoxic conditions by nearly 2-fold, as compared with the control vector transfected p53−/− cells, whereas WT cells transfected with the vectors expressing the mutant NPMΔC deficient in p53 binding were as sensitive to hypoxia as those transfected with control vectors. In p53−/− cells, overexpression of neither WT NPM nor the mutant NPMΔC had significant effect on hypoxia-induced cell death compared with the control vector transfected p53−/− cells. Taken together, these results indicate that NPM protects cells from hypoxia-induced cell death through a mechanism involving p53.

_Hypoxia-induced NPM Inhibits Phosphorylation of p53Ser-15._

Because we observed no increased protection against hypoxia-induced cell death in NPM-overexpressing p53−/− cells, we reasoned that NPM protects against hypoxic cell death by inhibiting p53 activity. It has been shown that hypoxia induces phosphorylation at the Ser-15 residue of p53 (P-p53Ser-15), which is critical for p53 transactivation and subsequent apoptotic signal transduction (27, 28). We thus determined whether hypoxia-induced NPM affected p53 activity. Normal human fibroblasts transfected with control (empty vector; Fig. 4A, lanes 1 and 2), WT NPM (lanes 3 and 4), mutant NPMΔC (lanes 5 and 6) vectors, or siNPM (lanes 7 and 8) duplexes were incubated in normoxia or hypoxia for 16 h. Hypoxia induced p53Ser-15 phosphorylation, resulting in the up-regulation of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 (Fig. 4A, lane 2). Strikingly, overexpression of NPM significantly reduced P-p53Ser-15 and p21 (lane 4), whereas overexpression of the mutant NPMΔC, which lacks the p53-interacting domain (22), failed to inhibit p53 activation (lane 6). Suppression of NPM expression by siNPM increased the level of P-p53Ser-15 and p21 (lane 8). We next asked whether inhibition of p53 activation was due to interaction between the two proteins. Indeed, we found that hypoxia increased association of endogenous NPM with p53 (Fig. 4B, compare lanes 2 and 4). It appeared that NPM interacted with both total p53 and P-p53Ser-15. While forced expression of exogenous NPM under hypoxic conditions did not further enhance its association with p53 (Fig. 4B, lower panel, lane 5), it did significantly reduce the bound P-p53Ser-15 (Fig. 4B, upper panel, lane 5). Overexpression of the mutant NPMΔC had no effect, consistent with its defect in interaction with p53 (22). Transfection of the cells with either siNPM (lane 7) or the control siRNA (lane 6) did not appear to have significant effect on the NPM-p53 interaction, although we observed increased P-p53Ser-15 bound to the endogenous NPM (Fig. 4B, upper panel, lane 8). These results collectively demonstrate that NPM plays a role in regulation of hypoxia-induced p53 activity, possibly by directly binding to the tumor suppressor.

_Hypoxia Induces NPM Expression._

Hypoxia plays an important role in many pathological processes such as ischemic stroke and tumor progression. Cells respond to hypoxia by expressing a variety of gene products to adapt to altered environments or to exploit for a survival/proliferative advantage (1). Here we have demonstrated that hypoxia induces the expression of NPM, a protein frequently overexpressed in a variety of human malignancies. We have also shown that the NPM promoter encodes a functional HRE that can be activated by hypoxia or by forced expression of HIF-1α. Suppression of NPM expression by siRNA targeting NPM increases hypoxia-induced apoptosis. Induction of NPM in response to hypoxia has important physiological relevance because hypoxic stress requires inductive expression of anti-apoptotic genes like NPM to direct the cell toward the survival/proliferative state instead of the apoptotic state. In addition, our study demonstrates that hypoxic cells overexpressing NPM are more resistant to apoptosis possibly through inhibition of p53, thus providing proof of concept evidence that the pathological elevations of NPM found in cancers and leukemias are important for maintaining cell survival and resistance to apoptosis.

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Addendum—During preparation of this manuscript, Maiguel et al. (29) also reported that NPM interacts with p53 and inhibits phosphorylation of p53Ser-15.

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