Hypoxia in the thymus: role of oxygen tension in thymocyte survival

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Hale, Laura P., Rod D. Braun, William M. Gwinn, Paula K. Greer, and Mark W. Dewhirst. Hypoxia in the thymus: role of oxygen tension in thymocyte survival. Am J Physiol Heart Circ Physiol 282: H1467–H1477, 2002. —Our previous studies using oxygen microelectrodes showed that the thymus is grossly hypoxic under normal physiological conditions. We now have investigated how oxygen tension affects the thymus at the cellular and molecular level. Adducts of the hypoxia marker drug pimonidazole accumulated in foci within the cortex and medulla and at the corticomediastinal junction, consistent with the presence of widespread cellular hypoxia in the normal thymus. Hypoxia-associated pimonidazole accumulation was decreased but not abrogated by oxygen administration. Genes previously reported to be induced by hypoxia were expressed at baseline levels in the normal thymus, indicating that physiological adaptation to hypoxia occurred. Despite changes in thymus size and cellularity, thymic PO2 did not change with age. Combined assays for hypoxia and cell death showed that hypoxia achieved using either hypoxic gas mixtures or high-density culture in normoxia decreased spontaneous thymocyte apoptosis in vitro. Taken together, these data suggest that regulatory mechanisms exist to maintain thymic cellular hypoxia in vivo and that oxygen tension may regulate thymocyte survival both in vitro and in vivo.

Apoptosis; gene regulation

Hypoxia has long been known to cause cell death, but the molecular mechanisms of hypoxia-induced cell death are just now being elucidated. Hypoxia can cause cell death by apoptosis as well as by necrosis (35). Apoptosis is characterized histologically by nuclear condensation and fragmentation with degradation of chromosomal DNA into ~180-bp oligomers, condensation of cytoplasm, and formation of apoptotic bodies. Necrosis results from ATP depletion and is characterized by increased cytoplasmic eosinophilia and vacuolation due to degradation of cytoplasmic RNA and protein, mitochondrial swelling, discontinuities in plasma and organelle membranes, and nuclear changes eventually resulting in loss of nuclei (7). The process of apoptosis is an active, highly regulated process, whereas necrosis has been considered to be more of a passive response to direct cellular injury. Expression of Bcl-2 or Bcl-XL has been shown to prevent hypoxia-induced apoptosis in a dose-dependent manner (35). Thus differences in the relative proportions of hypoxia-induced apoptosis and necrosis that have been observed between different cell types may relate to their relative expression of proapoptotic and antiapoptotic factors including Bcl-2 or Bcl-XL.

Exposure to hypoxia typically causes changes in expression of genes that function to either increase tissue oxygen delivery or influence cellular survival in a low oxygen environment. Examples of hypoxia-responsive genes include glucose transporters, glycolytic enzymes, and 78- and 94-kDa glucose-regulated proteins (GRP78 and GRP94, respectively) as well as vascular endothelial growth factor (VEGF) (11, 31). Hypoxia changes gene expression primarily through the action of the hypoxia-inducible factor-1 (HIF-1) transcriptional activator (32, 34). HIF-1 is a basic helix-loop-helix heterodimeric complex composed of a novel HIF-1α subunit and HIF-1β, originally identified as the aryl hydrocarbon receptor nuclear translocator. Although HIF-1α is produced constitutively, it has a very short half-life. Hypoxia prevents its degradation and increases its DNA binding, leading to rapid accumulation of HIF-1 complexes, which induce activation of hypoxia-responsive genes (15). HIF-1 activity is also modulated by CO and nitric oxide (NO), which can affect HIF-1 binding to DNA without affecting HIF-1α protein expression (31). Expression of the cell death factor Bcl2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) has recently been shown to be HIF-1 dependent in both tumor cell lines and normal tissues (14, 36).

Another hypoxia-responsive gene, heme oxygenase (HMOX-1), has a binding site for HIF-1α, but its expression is not reduced in HIF-1α-deficient cells under hypoxic conditions. Thus HMOX-1 expression is thought to be regulated by factors other than HIF-1, but these regulators have not yet been identified (33). Other transcription factors such as nuclear factor

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(NF)-κB, activating protein-1, Egr-1, and C/EBP-β have also been shown to be activated by hypoxia (18). NF-κB induces a number of genes, including inducible NO synthase, cyclooxygenase-2 (COX2), and antiapoptosis tumor necrosis factor receptor-associated proteins 1 and 2 (TRAF1 and TRAF2, respectively), that may play a role in the response to hypoxia (23). However, because many diverse stimuli are now known to induce the activity of NF-κB (20), it is important to recognize that enhanced expression of any particular gene may be the result of cooperation between multiple transcription factors. Induction of hypoxia-responsive genes serves to promote erythropoiesis, angiogenesis, and vasodilation to facilitate $O_2$ delivery to the affected tissues and also decreases $O_2$ utilization by increasing anaerobic energy generation through glycolysis. Whether these genes would be similarly induced in cells adapted to hypoxia or when hypoxia is beneficial to cell or tissue function has not previously been described.

Tissue hypoxia has traditionally been measured using oxygen-sensing microelectrodes, although newer methods such as fluorescent fiber-optic sensors are becoming available. We have recently shown that the $P_{O_2}$ measured in a given tissue depends strongly on the area sampled by each measurement and the averaging technique used (4). Immunohistochemical markers for hypoxia have gained popularity recently because they can assess hypoxia on a cellular level rather than providing an average $P_{O_2}$ over variously sized microregions as with oxygen microelectrodes and optical sensors. The 2-nitroimidazole hypoxia marker, 1-[(2-hydroxy-3-piperidinyl)propyl]-2-nitroimidazole hydrochloride (pimonidazole hydrochloride), has a high water solubility (0.4 M), readily traverses cell membranes in its oxidized form, and also has sufficiently low toxicity for clinical use in animals and humans (maximum tolerated in vivo dose 2 g/m$^2$ for a single dose or 0.75 g·m$^{-2}$·day$^{-1}$ for extended periods) (17). At $P_{O_2} < 10$ mmHg, pimonidazole forms irreversible covalent adducts with cellular proteins that can be detected immunohistochemically. The formation of pimonidazole adducts has been shown to depend on the cellular oxygen tension, independent of the pyridine nucleotide redox status of the cell (1). Thus pimonidazole may be a useful marker for cells that have experienced hypoxia with $P_{O_2} < 10$ mmHg (29). An increasing number of studies have shown that accumulation of pimonidazole as a marker for physiological hypoxia and measured the expression of a panel of hypoxia-responsive genes in the thymus in vivo. We report an in vitro assay for cellular hypoxia that should be widely applicable to a variety of cultured cell types and determined effects of oxygen on thymocyte survival in vitro. Finally, we studied $P_{O_2}$ within the thymus as a function of age.

MATERIALS AND METHODS

Animals and cell culture. The in vivo measurements of oxygen tension used Balb/C mice. C57BL/6 mice were also used for some in vitro studies. All animal studies were approved by the Duke University Institutional Animal Care and Use Committee. Where indicated, mice were exposed to 100% $O_2$ in positive pressure chambers with a flow rate of 1 l/min of humidified 100% $O_2$. Animals were euthanized by an overdose of pentobarbital sodium. Thymocyte suspensions were obtained by gently pressing thymus tissues through a mesh screen. Thymocytes were cultured in polypropylene culture tubes at the indicated cell densities in RPMI 1640 + 10% fetal calf serum (FCS) + 5.5 × 10$^{-5}$ M 2-mercaptoethanol. Normoxic conditions used ambient air supplemented with 5% CO$2$. Hypoxic conditions used gas mixtures of 1% $O_2$-5% CO$2$-94% N$2$ (National Specialty Gases; Research Triangle Park, NC) in a Bactron Anaerobic Chamber (Sheldon Manufacturing; Cornelius, OH) to provide a nominal chamber $P_{O_2}$ of 6–10 mmHg. B16F10 (B16) murine melanoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in DMEM + 10% fetal bovine serum at 37°C in an atmosphere supplemented with 5% CO$2$.

In vivo $P_{O_2}$ measurements. Recessed-tipped oxygen microelectrodes were prepared, calibrated, and used as previously described (4, 22). Mice anesthetized with an intraperitoneal injection of 80 mg/kg pentobarbital sodium were placed on their backs on heated water blankets. The thymus was exposed via mediastinotomy, with care taken to avoid pneumothorax, and was kept moist by topical application of saline. A small incision was made in the forelimb, and an Ag/AgCl reference electrode was sutured into the subcutis. The oxygen microelectrode was advanced into the thymus using a micromanipulator, and $P_{O_2}$ was recorded for 10 s at 50-μm steps for a total distance of 1,000–2,000 μm. The microelectrode was then withdrawn, and the process was repeated for a total of three to four tracks. The total measurements made per thymus ranged from 62 to 155. At the end of the recording time, the mouse was euthanized by overdose of pentobarbital sodium, and recordings were continued for at least 5 min after euthanization to obtain a true in vivo zero as previously described (4, 8).
Hypoxia marker studies. Pimonidazole hydrochloride (Natural Pharmacia; Belmont, MA) was prepared as a 100 μg/ml stock solution in 0.9% saline. Mice were injected with 70 mg/kg pimonidazole intraperitoneally 3 h before death to allow for clearance from normal tissues and to avoid pimonidazole adduct formation at the time of euthanasia. Organs were removed within 2 min of death, immediately fixed in 10% neutral buffered formalin for 24–48 h, and then processed into paraffin blocks. Four-micrometer-thick sections were immunostained using standard protocols, including deparaffinization, blocking of endogenous peroxidase activity (0.6% H2O2 in absolute methanol, 15 min), and diluted goat serum blocking. The slides were then sequentially incubated at 37°C with anti-pimonidazole primary antibody (polyclonal rabbit, the kind gift of J. A. Raleigh) or normal rabbit immunoglobulin, biotinylated goat anti-rabbit immunoglobulin secondary antibody, and avidin-biotin horseradish peroxidase (HRP) complexes (VectaStainABC, Vector Laboratories; Burlingame, CA), with intervening PBS washes. Bound antibody was detected with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Slides were counterstained with hematoxylin, dehydrated, and permanently mounted.

For in vitro studies with pimonidazole, murine thymocytes were incubated with varying concentrations of pimonidazole in RPMI 1640 (GIBCO-BRL; Grand Island, NY) + 10% FCS for 70 min before harvest. Cells were washed in PBS and then permeabilized by overnight fixation in 70% ethanol. Fixed cells were applied to glass slides using a cytocentrifuge and stained with anti-pimonidazole antibody as described above. Alternatively, cells were reacted with anti-pimonidazole polyclonal antibody, washed, and then reacted with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin. Positive cells were detected by flow cytometric analysis using a FACStar Plus (BD Biosciences; San Jose, CA), with at least 10,000 cells analyzed for each set of conditions tested.

Apoptosis and cell cycle assays. The percent apoptosis in thymocyte cultures was measured using the annexin V assay from Immunotech (Marseille, France). Flow cytometry was completed within 10 min of cell harvest and staining. Apoptotic cells were defined as annexin V positive and propidium iodide negative. Necrotic cells were defined as positive for both annexin V and propidium iodide. Viable cells do not react with either reagent. Cell cycle analysis was performed by flow cytometric staining of ethanol-fixed cells with propidium iodide. Apoptotic cells were defined as cells that contained <2n DNA and were present within the sub-G0 peak.

Studies of gene expression. Thymus and control tissues used in gene expression studies were removed under deep anesthesia immediately before animal death by barbiturate overdose and immediately snap-frozen as a precaution to minimize any possible postmortem induction of hypoxia-responsive genes. Total RNA was obtained using the RN Easy kit (Qiagen; Valencia, CA). Two micrograms of RNA were reverse transcribed using SuperScript (GIBCO-BRL), and the resulting cDNA was subjected to PCR for the indicated number of cycles using Platinum Taq (GIBCO-BRL). Primers were selected to cross intron-exon boundaries and did not amplify genomic DNA. Primer sequences and the resultant product sizes were as follows: GRP78 (257 bp), 5′-GCA GTT GTT ACT ACT GCA ACA-3′ and 5′-CAT GTA GTT AGC TAC TCC ATT-3′ (bp 597–614 and 854–838, GenBank D78645); HMOX-1 (269 bp), 5′-CGT GAG TAC TAC AAG AAC-3′ and 5′-GGC TTT ACA TAG TGC TGC-3′ (bp 219–236 and 488–461, GenBank NM 010442); Cox2 (206 bp), 5′-ACC AGT ATG AGT GTG ACT-3′ and 5′-GAT CTG GAT GAC ACA-3′ (bp 237–255 and 444–427, Genbank NM 011198); TRAF2 (308 bp), 5′-TCG GCC TTT ACA GAT AAC-3′ and 5′-CTA CAG GAG CTT AAG GGA-3′ (bp 312–329 and 619–602, GenBank L53033); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 214 bp), 5′-TCG TCC CTA AGA CAA AAT G-3′ and 5′-TGA CAA GCT CAT CTC C-3′ (bp 31–49 and 244–227, GenBank M32599); β-actin (220 bp), 5′-GAA GCT GTG CTA TGT TGC-3′ and 5′-CGT CAC ACT TCA TGA TGG-3′ (bp 722–739 and 939–922, GenBank X003672); and BNP3 (280 bp), 5′-CAG CAT GAA TCT GGA CCA-3′ and 5′-TGC TGA GAG TAG CTG TGC-3′ (bp 216–233 and 495–478, GenBank NM 009760). For Northern blots, 15 μg RNA from each sample were electrophoresed and transferred to nitrocellulose using standard procedures. Blots were probed with a PCR-generated probe corresponding to bp 216–495 of the BNP3 gene using the NorthSouth Direct HRP Labeling and Detection kit (Pierce; Rockford, IL) according to the manufacturer’s instructions.

RESULTS

Pimonidazole adducts accumulate in normal thymus tissues in vivo. Our previous studies have shown that the mean PO2 within the thymus was <10 mmHg when measured using microelectrodes, suggesting that the thymus may be hypoxic in vivo (4). However, because microelectrodes and oxygen sensors average PO2 over a microregion, it is important to determine whether similarly low PO2 levels exist at the cellular level and how the oxygen tension varies anatomically within the tissue itself. Therefore, we administered the hypoxia marker drug pimonidazole in vivo to normal 6- to 8-wk-old mice breathing room air and analyzed the distribution of thymic cells immunohistochemically reactive with antibodies that recognize pimonidazole adducts (Pimo*). We found that the normal murine thymus contained large numbers of cells reactive with anti-pimonidazole antibody (Fig. 1A, B, and C), indicating that cellular PO2 levels of <10 mmHg were present in these cells in vivo. The punctate appearance at low magnification (Fig. 1B) was due to individual whole cell staining, as seen in the higher magnification view of Fig. 1C. Serial sections reacted with normal rabbit immunoglobulin were negative (Fig. 1A), as were thymus tissues from mice that did not receive pimonidazole in vivo (results not shown). The intensity of pimonidazole staining and numbers of thymocytes that were reactive with anti-pimonidazole antibody increased with distance from thymic blood vessels (Fig. 1C). Thymic blood vessels as well as the thymocytes directly surrounding them were typically nonreactive with anti-pimonidazole antibody, serving as an additional internal control that confirms the previously reported specificity of pimonidazole immunohistochemical staining for hypoxic cells. Although the thymocytes in the subcapsular cortex were rarely pimonidazole positive, thymocytes faintly reactive with anti-pimonidazole antibody were present throughout the remainder of the cortex. The majority of the cells in the thymic medulla were also Pimo* positive. Medullary thymocytes had a slightly greater staining intensity than the majority of the cortical thymocytes. Occasional thymic epithelial cells were also identified as...
faint to moderately pimonidazole positive. Additional foci of thymocytes that were very strongly reactive with anti-pimonidazole antibody were present in these mice, generally located in the midcortex or near the corticomedullary junction (Fig. 1B). These foci covered from 5 to 10% of the thymic area studied in normal mice breathing room air.

As an additional control, we also stained spleen sections from pimonidazole-treated mice breathing room air for the presence of pimonidazole adducts. Previous studies have shown that mean PO2 values in the spleen average ~20 mmHg, with PO2 <10 mmHg in ~10% of measurements (4). We found that cells reactive with anti-pimonidazole antibody were concentrated in the red pulp, directly under the splenic capsule (results not shown), consistent with the well-described distribution of blood vessels and blood flow within the spleen and in numbers consistent with our previous measurements of splenic PO2. The majority of splenic lymphocytes were nonreactive with anti-pimonidazole antibody, again consistent with the specificity of pimonidazole staining for hypoxic cells.

Hypoxia-responsive genes are not induced in normal thymus in vivo. Our oxygen tension measurements (4) and our immunohistochemical hypoxia marker studies both indicate that the normal thymus is a hypoxic organ. Hypoxia has been shown to alter gene expression, particularly of genes whose products may decrease oxygen utilization and/or increase oxygen supply. To determine whether the normal hypoxic status of the thymus induces expression of genes previously documented to be hypoxia responsive, we analyzed the normal murine thymus for expression of GRP-78, HMOX-1, COX2, TRAF2, and GAPDH as well as for β-actin as a control using semiquantitative RT-PCR assays. Expression of GRP-78, HMOX-1, COX2, TRAF2, and GAPDH have all previously been shown to be induced by hypoxia (11, 23, 31, 33). The baseline expression of each of these genes was determined using tissues from mice that had breathed 100% O2 for 4 h before tissue harvest. The lung and spleen were chosen as nonhypoxic control tissues. The lung was expected to be the most highly oxygenated tissue within the body and normal mice breathing 100% O2 would be expected to have no pulmonary hypoxia. We have previously documented that the spleen is not generally hypoxic in mice breathing room air (Ref. 4 and data above), and the spleen has a mean PO2 intermediate between that of the thymus and lung. The level of expression of GRP-78, HMOX-1, COX2, TRAF2, GAPDH, and β-actin mRNA for the thymus, lung, and spleen from mice breathing room air or 100% O2 is

Fig. 1. Pimonidazole adducts characteristic of tissue hypoxia can be detected in the thymus. The thymus from mice treated in vivo with the hypoxia marker drug pimonidazole while breathing either normal room air (normoxia; A–C) or 100% O2 (D–F) for 4 h were reacted with anti-pimonidazole polyclonal antibody (Ab) as described in MATERIALS AND METHODS. The brown color indicates a positive reaction. The thymus from mice breathing room air (B and C) shows generalized weak-to-moderate reactivity with anti-pimonidazole Ab, with additional strongly reactive foci. Immunoreactivity generally increases with increasing distance from blood vessels (v). Anti-pimonidazole reactivity was reduced, but not abrogated, in the thymus from mice exposed to 100% O2 for 4 h before tissue harvest (E and F). No reactivity was seen when these tissues were reacted with control Ab (A and D) or when the thymus from mice not treated with pimonidazole was reacted with anti-pimonidazole Ab (data not shown). Bar = 50 μm.
shown in Fig. 2A. The baseline level of expression of hypoxia-responsive genes in each of these organs is represented by results from mice breathing 100% O₂ (Fig. 2A, lanes 4–6). Baseline COX-2 expression was faint to absent in the spleen, and HMOX-1 was more highly expressed in the spleen compared with the thymus and lung. As expected, hypoxia-responsive genes were not induced (i.e., the level of expression was similar to that in mice breathing 100% O₂) in the lung or spleen from mice breathing room air (Fig. 2A, middle and right, lanes 1–3). This confirms previous results demonstrating that these organs are not hypoxic under normal conditions. However, expression levels were similar, and thus none of these genes were induced in the thymus of mice breathing room air (Fig. 2A, left, lanes 1–3) relative to the thymus from mice breathing 100% O₂ (Fig. 2A, left, lanes 4–6) despite the observed severe tissue and cellular hypoxia documented in the thymus of mice breathing room air by both microelectrode and hypoxia marker studies.

To further investigate the apparent lack of induction of hypoxia-responsive genes in the normal thymus despite its hypoxia documented by microelectrode and pimonidazole assays, we analyzed expression of the BNIP3 gene by Northern blot. BNIP3 has previously been documented to be hypoxia responsive, with very low baseline levels under normoxic conditions and strong induction at both the mRNA and protein levels by hypoxia in 15 of 18 human cell lines tested (36). We reasoned that this very low baseline expression would facilitate detection of hypoxia-induced gene expression. We found that BNIP3 mRNA was undetectable under normoxic conditions in the B16 murine melanoma cell line and was strongly induced by 18 h of hypoxia (Fig. 2B, lane 8) compared with normoxic controls (Fig. 2B, lane 7). As expected, BNIP3 expression was undetectable in the nonhypoxic control lung and spleen tissues (lanes 1–4).

Fig. 2. Hypoxia-responsive genes are not induced in the normal thymus. A: expression of indicated mRNAs {78-kDa glucose-regulated protein (GRP-78), heme oxygenase (HMOX)-1, cyclooxygenase (COX)-2, tumor necrosis factor receptor-associated protein (TRAF2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin} in the thymus, lung, and spleen of mice breathing either room air (lanes 1–3) or 100% O₂ (lanes 4–6) for 4 h was determined by RT-PCR amplification for 20, 25, or 30 cycles (left to right, respectively). Results are shown for 3 tissues of each type analyzed except for the lung, where lane 2 (room air) was not available for analysis. The lung and spleen are not hypoxic and show a corresponding lack of induction of hypoxia-responsive genes for mice breathing either room air or 100% O₂. Hypoxia-responsive genes are also expressed at similar levels in the thymus from mice breathing room air and those breathing 100% O₂, indicating lack of induction of these genes by the hypoxia observed in the thymus from mice breathing room air. Baseline levels are similar, and none of these genes were induced in the thymus from mice breathing room air (lanes 4–6) or 100% O₂ (lanes 1–3).

B: expression of Bcl-2/adenovirus E1B 19-kDa-interacting protein (BNIP3) was determined by Northern blot of tissues from mice breathing room air or 100% O₂ for 4 h and cell lines cultured under normoxic and hypoxic conditions for 18 h. Lane 1, lung (room air); lane 2, lung (100% O₂); lane 3, spleen (room air); lane 4, spleen (100% O₂); lane 5, thymus (room air); lane 6, thymus (100% O₂); lane 7, B16 cells (normoxic culture); lane 8, B16 cells (hypoxic culture). BNIP3 expression was strongly induced by hypoxia in B16 cells (lane 8 vs. lane 7). However, no BNIP3 expression was detectable in the thymus from mice breathing room air (lane 5) or 100% O₂ (lane 6). BNIP3 expression was similarly undetectable in the nonhypoxic control lung and spleen tissues (lanes 1–4). The positions of the ethidium bromide-labeled 28S and 18S rRNAs were used to estimate BNIP3 transcript size as 1.7 kb. The 18S band is shown as a loading control.
2B, lanes 7 and 8) (positive control). However, BNIP3 mRNA was undetectable in the thymus from mice breathing room air or 100% O2 (Fig. 2B, lanes 5 and 6) and in the control nonhypoxic lung and spleen tissues from mice breathing room air or 100% O2 (Fig. 2B, lanes 1–4).

Failure to induce expression of these hypoxia-responsive genes in the normal thymus in the face of well-documented hypoxia may reflect a physiological adaptation of the thymus to chronic hypoxia. If the normal state of thymic hypoxia is beneficial, then induction of hypoxia-responsive genes may be detrimental to thymic function. For example, expression of BNIP3 is associated with cell death in both hypoxia-exposed cells and transient transfectants (14, 36). It is possible that expression of hypoxia-responsive genes may be regulated differently (e.g., by posttranscriptional or other mechanisms) in the thymus, an organ that is hypoxic under normal conditions, in contrast to hypoxic tumors or to other previously described well-oxygenated normal organs (14, 18, 36). Alternatively, apparent lack of induction of hypoxia-responsive genes in the thymus from mice breathing room air relative to baseline expression in mice breathing 100% O2 may simply reflect an inability of inhaled 100% O2 to abrogate thymic hypoxia.

Treatment with 100% O2 increases mean thymic PO2 but does not totally relieve thymic hypoxia in vivo. Many organs, most notably the brain, regulate blood flow so that oxygen supply is very closely matched to oxygen consumption. The mechanisms are complex and still poorly understood; however, this regulation is thought to protect against potential adverse effects of excess oxygen exposure, including generation of reactive oxygen species and oxidative damage to tissue. How the flow of blood is regulated relative to oxygen demand by the thymus is unknown. If tissue PO2 levels contribute to regulation of thymic blood flow, we would expect that hyperoxygenation would result in decreased blood flow, with minimal change in overall tissue oxygenation. If tissue PO2 levels do not contribute to regulation of thymic blood flow, we would expect to see increased thymic PO2 and absence of pimonidazole staining in the thymus from mice undergoing hyperoxygenation.

To investigate the effect of increased inhaled O2 on thymic oxygenation, we measured tissue PO2 levels using microelectrodes in normal 6- to 8-wk-old mice, first while breathing room air (21% O2) and then after a switch to 100% O2 delivered via face mask. Thymus PO2 measurements generally increased within 1 min of administration of 100% O2, from 14 ± 3.7 mmHg on room air to 135 ± 45 mmHg on 100% O2 (means ± SE, n = 6; Fig. 3). However, the rate of PO2 increase and its duration were highly variable from animal to animal. Two animals showed minimal to no change in thymic PO2 with 100% O2. In some animals, thymic PO2 initially increased in response to 100% O2 and then decreased without a change in the fraction of inhaled O2. This pattern of variability is most consistent with additional regulation of PO2 at the level of thymic blood flow.

Although the mean thymic PO2 increases rapidly with oxygen administration, pimonidazole adducts can still be detected immunohistologically in the thymus, even when pimonidazole was administered after 1 h of breathing 100% O2 (Fig. 1, D–F). However, the frequency of foci containing large numbers of pimonidazole-positive thymocytes was decreased in mice breathing 100% O2 relative to those breathing room air (Fig. 1E), and the intensity of pimonidazole staining was decreased. However, individual hypoxic cells can still be observed even in regions adjacent to multiple blood vessels (Fig. 1F). Taken together with the microelectrode data above, these studies demonstrate that PO2 varies anatomically within the thymus and that increased oxygen delivery decreases thymic hypoxia but is insufficient to totally eradicate hypoxia at the cellular level. Particularly when taken together with the lack of induction of hypoxia-responsive genes, these results suggest that regulatory mechanisms exist to maintain thymic hypoxia.

Thymic PO2 in vivo does not change with age. The oxygen tension present in the thymus reflects both oxygen supply and demand. Thymocytes develop in close association with thymic epithelial cells and are separated from thymic blood vessels by variable amounts of connective tissue, mature lymphocytes, and a basement membrane. During age-related thymic atrophy in mice, the thymic area involved in thymopoiesis decreases, such that the remaining thymocytes are in closer proximity to thymic blood vessels and may thus potentially experience a higher local PO2 if thymic...
blood flow remains constant. Age-related decreases in the numbers of developing thymocytes present in the thymus would also be predicted to decrease oxygen utilization and thus to potentially increase thymic PO2.

To determine how thymus PO2 changes with age, we measured thymic oxygen tension in vivo in mice of varying ages using an oxygen microelectrode. As shown in Fig. 4, mean thymic PO2 was 10.4 ± 1.5 mmHg for 5- to 6-wk-old mice (n = 11) vs. 9.4 ± 2.2 mmHg for 40- to 41-wk-old mice (n = 5; means ± SE, \( P = 0.10 \)). Median thymic PO2 was 8.2 ± 1.4 and 7.0 ± 2.3 mmHg (means ± SE, \( P \) = not significant) for 5- to 6-wk-old vs. 40- to 41-wk-old mice, respectively. These studies demonstrate that thymic PO2 does not change significantly with age despite age-related changes in thymus size, histology, and thymopoietic activity. They further suggest that regulatory mechanisms exist during aging to maintain thymic hypoxia at the whole organ level in addition to regulation at the cellular level described above.

**In vitro assay for hypoxia.** We and many others have noted rapid and abundant apoptosis of thymocytes cultured in vitro at ambient oxygen concentrations. If the thymus is normally adapted to hypoxic conditions, then exposure to higher levels of oxygen may be detrimental to thymocytes. We wanted to determine whether limiting the oxygen to levels similar to what we observed in the thymus in vivo could improve the survival of cultured murine thymocytes in vitro. However, to allow us to determine the extent of thymocyte hypoxia under normal culture conditions, we first needed to develop an assay for hypoxia in vitro.

It should be possible to achieve in vitro hypoxia by either increasing oxygen consumption within cultures or by decreasing atmospheric oxygen content. We first analyzed pimonidazole immunoreactivity in thymocytes cultured at high density (100 × 10⁶ thymocytes/ml) for 24 h under normoxic conditions. We hypothesized that normal oxygen utilization by these crowded cells could lead to hypoxia in the culture medium at ambient oxygen concentrations. Optimal pimonidazole loading occurred when thymocyte cultures were pulsed with 400–800 \( \mu \)g/ml pimonidazole for 70 min (Fig. 5A). No increases in numbers of positive cells or in intensity of immunoreactivity with anti-pimonidazole antibody was achieved with longer loading times (data not shown). The percentage of pimonidazole-positive cells was greatly decreased when thymocytes were cultured at lower cell densities (Fig. 5B), consistent with consumption-induced hypoxia in the higher density cultures. However, the number of pimonidazole-positive thymocytes under the high-density culture conditions was consistently less than one-half of the total thymocytes.

An inability to achieve global hypoxia in high-density cultures may reflect properties of the thymocytes themselves or may reflect differences in oxygen tensions in local microenvironments within the culture. To address this first possibility, we determined whether the pimonidazole immunoreactivity of thymocytes differed as a function of their cellular proliferation status. Thymocytes cultured at high density under normoxic conditions for 24 h were pimonidazole pulsed and then simultaneously analyzed for hypoxia and DNA content using anti-pimonidazole and propidium iodide double staining of permeabilized cells. We found that very few pimonidazole-positive cells were present in the apoptotic cell fraction (open bars in Fig. 5C). Cells already committed to apoptosis at the time of pimonidazole addition were not expected to become pimonidazole positive, because formation of pimonidazole-protein adducts requires active cytochrome P-450 enzymes (30). In contrast, large fractions of both G0/G1 and G2/S/M phase thymocyte populations were pimonidazole positive (Fig. 5C). Furthermore, because hypoxia has previously been shown to prevent proliferation (30), it is likely that the pimonidazole-positive thymo-

![Fig. 4. Thymic PO2 does not change with age. The median cumulative frequencies of the given PO2 ± the interquartile range for the set of mice at each age are shown (A: n = 11, 5–6 wk; B: n = 5, 40–41 wk). A and B, insets: global PO2 histograms, i.e., all of the PO2 values measured in all the mice. PO2 values did not differ statistically between the groups tested.](http://ajpheart.physiology.org/)
cytes in G2/M/S phases became hypoxic after beginning their progression through the cell cycle. Taken together, these data suggest that variations in percentages of pimonidazole-positive viable thymocytes of similar proliferation status within cultures are most likely due to local fluctuations in the oxygen microclimate within the bulk culture itself. Clearly, very low PO2 is generated in some regions during high-density culture of thymocytes under normoxic conditions.

Hypoxia reduces thymocyte apoptosis but increases thymocyte necrosis in vitro. To determine whether limiting the oxygen to levels similar to what we observed in the thymus in vivo could improve the survival of cultured murine thymocytes in vitro, we examined thymocyte survival in cultures with varying cell densities under both normoxic and hypoxic conditions. The mechanism of thymocyte death was determined to be via necrosis or apoptosis using combined annexin V-propidium iodide exclusion flow cytometric assays. The percentage of apoptotic cells measured using this assay was similar to that measured using the DNA content assay for thymocytes cultured under similar conditions. Hypoxia achieved either using the hypoxic chamber or high-density culture in normoxia decreased the fraction of the thymocyte population undergoing apoptosis (Fig. 6A, open bars). However, the fraction of thymocytes undergoing necrosis (Fig. 6A, gray bars) increased under hypoxic conditions, with little net change in the percentage of viable thymocytes (Fig. 6A, solid bars) in hypoxia vs. normoxia.

The annexin V-propidium iodide exclusion assay that we used allows identification of populations of cells in early and midstage apoptosis. Cells in the final stages of apoptosis are positive for annexin V and also fail to exclude propidium iodide, similar to cells that have undergone necrosis (2). To exclude the possibility that the increased number of cells scored as necrotic resulted from faster progression through apoptosis under hypoxic conditions, we compared the relative proportions of apoptotic and necrotic cells in normoxic and hypoxic cultures as a function of time. As shown in Fig. 6B, similar levels of apoptosis (open bars) and necrosis (gray bars) were observed under hypoxic conditions, with little net change in the percentage of viable thymocytes (Fig. 6A, solid bars) in hypoxia vs. normoxia. The numbers of necrotic cells increased markedly at 24 h in hypoxic cultures (Fig. 6B, light gray bars). Analysis of the corresponding flow cytometric data (Fig. 6C) indicates that this increase in necrotic thymocytes (top right) occurs without an increase in prior early stage apoptotic cells (bottom right). Thus the increased necrosis seen in hypoxic cultures most likely results from anoxia rather than from more rapid progression of hypoxic cells through apoptosis.

DISCUSSION

In this study, we used the hypoxia marker pimonidazole to show that individual cells within the thymus are normally hypoxic in vivo, i.e., they experience PO2 <10 mmHg. We also showed that thymus hypoxia persists at the cellular level despite oxygen administration and at the organ level despite age-related anatomic changes that would be predicted to increase PO2. Furthermore, thymocyte hypoxia was associated with decreased apoptosis in vitro. Previous studies have
shown that dexamethasone-induced apoptosis of rat thymocytes is inhibited in hypoxic atmospheres (0.5–5% O₂) and increased in high oxygen atmospheres (95% O₂) (37). The dexamethasone-induced apoptosis of murine thymocytes was also shown to be inhibited by hypoxia (24, 38). Our studies further confirm the role of oxygen in regulating spontaneous thymocyte apoptosis and also document new methods for demonstrating hypoxia in thymocytes both in vitro and in vivo. Taken together, these studies suggest that decreased thymic oxygen tension may increase thymocyte survival and that the thymus has evolved mechanisms to closely regulate oxygen supply and demand in vivo.

Several previous studies have suggested that oxygen and reactive oxygen species may decrease murine thymocyte survival in vivo. Exposure to 2.8 atm 100% O₂ (PO₂ of 1,884 mmHg) for 4 h daily over 3 days has been shown to markedly decrease CD4⁺ CD8⁺ immature thymocytes from 85.2% to 21.2% (40). More mature single positive (CD4⁺ or CD8⁺) thymocytes and Thy1⁺ T cells in the spleen were less sensitive to increased oxygen (40). Exposure to 0.7 parts per million ozone for 20 h/day caused a decrease in thymus weight for days 3–9, but the thymus weight returned to baseline by day 14 (10). Decreased thymus weight was associated with an up to 85% reduction in thymocyte number, and histological examination showed marked depletion of the thymic cortex, where immature double positive thymocytes are normally located. Adrenalectomized animals had a decrease of 30% in thymus weight vs. 60% for control and sham adrenalectomized animals when exposed for 4 days, indicating that stress and systemic corticosteroid secretion cannot by themselves account for the ozone-induced loss in thymocyte cellularity (10). Continuous exposure to 100% O₂ also greatly decreases thymocyte survival in vivo. Whole thymus weights decreased to 72 ± 10% of control at 72 h and 42 ± 3% of control after 96 h of exposure to 100% O₂, with corresponding thymocyte numbers of

Fig. 6. Hypoxia decreases apoptosis in thymocyte cultures but simultaneously increases ischemic necrosis. Thymocytes cultured under the indicated conditions were reacted with annexin V and propidium iodide and immediately analyzed by flow cytometry. Viable cells are indicated by dark gray bars, necrotic cells by light gray bars, and apoptotic cells by open bars. In A, bars labeled N and H indicate results from culturing under normoxic and hypoxic conditions, respectively. A: hypoxia generated by using hypoxic gas mixtures (hypoxia) or high-density cell culture (100 × 10⁶ cells/ml; normoxia) for 24 h decreases thymocyte death by apoptosis while variably increasing death by necrosis. Data shown are from a single experiment representative of >3 performed. P < 0.001 for differences between percentages of apoptotic and necrotic cells cultured under normoxia or hypoxia. B: hypoxia-induced changes in the percentages of thymocyte cell death by apoptosis vs. necrosis in thymocytes cultured at 10 × 10⁶ cells/ml occur after >8 h of culture in a hypoxic environment. C: increased numbers of dual annexin V/propidium iodide-positive cells (necrotic; top right of each plot) seen in thymocytes cultured at 10 × 10⁶ cells/ml in hypoxia result from direct progression from viable annexin V/propidium iodide dual-negative cells (bottom left of each plot), without passage through an annexin V single-positive (apoptotic) stage (bottom right of each plot). t, Time.
respectively (12). However, mice with prolonged exposure to 100% O2 also suffered severe pulmonary toxicity, leading to 50% mortality by day 5 (12).

Oxygen has also been shown in other studies to play a role in thymocyte differentiation and to increase thymocyte survival. The antioxidants N-acetyl-L-cysteine and butylated hydroxyanisole cause a dose-dependent arrest of thymocyte differentiation toward αβ-T cells in day 14 murine fetal thymic organ cultures (16). This is associated with a profound decrease in the nuclear content of NF-κB and in T-cell-specific factor 1(α) [TCF1(α)] transcription factor activity by electrophoretic mobility shift assay. Elevations in O2 in standard suspension cultures cause differentiation toward αβ-T cells and increase NF-κB (16). In mice of 18–24 mo of age, there was an increase in thymic cells with single 45-min exposure at 0.88 atm O2 (normal = 0.2 atm) but no change in thymocyte numbers with exposure to hyperbaric O2 for 2 h each day for 20 days (19). This study hypothesizes that an age-dependent increase in hypoxia may be responsible for immune system aging (19); however, our data show very clearly that thymic oxygen content does not change with age and thus does not support this hypothesis. We feel the increased thymus cellularity observed by Lee et al. (19) was more likely an acute response to hyperoxia, because it was not sustained with repeated exposure.

Thymocyte export from the thymus depends on the balance between cellular proliferation and cell death by both apoptosis and necrosis. The rate of apoptosis depends on the balance of proapoptotic and antiapoptotic factors. Hypoxia has been shown to cause cell death by apoptosis as well as by necrosis (35). Our studies indicate that thymus tissues that are hypoxic in vivo do not express detectable amounts of BNIP3, a gene product that rapidly induces cell death and is induced by hypoxia in many cell lines and normal tissues (14, 36). Hypoxia has been shown to select for tumor cells with defects in apoptosis, such as those with loss of p53 or overexpression of Bcl-2 (13). Whether similar selection processes occur for thymocytes due to their normally hypoxic environment requires additional studies.

The mechanisms by which hypoxia may aid thymocyte survival are not well understood. When quiescent thymocytes are stimulated to divide, they switch from oxidative phosphorylation to glycolysis as a means of ATP production. This change has been suggested to occur to reduce generation of reactive oxygen species that might damage replicating DNA (3, 31). However, it may also be the natural result of regulatory processes that limit thymocyte oxygen supply. Very recent studies indicate that hypoxic culture completely inhibits dexamethasone-induced apoptosis of murine thymocytes but does not affect apoptosis induced by anti-CD95 treatment (38). These studies indicate the presence of two distinct forms of thymocyte apoptosis: an oxygen-dependent pathway (e.g., dexamethasone induced) and an oxygen-independent pathway (e.g., anti-CD95 induced). The oxygen-dependent step in dexamethasone-induced apoptosis lies upstream of caspase-3-like protease activation, and the two pathways converge upstream of mitochondrial changes (38). Our data show that hypoxia generated in a hypoxia chamber or by high-density culture inhibits but does not prevent spontaneous thymocyte apoptosis, suggesting that both apoptotic pathways are active during spontaneous thymocyte apoptosis. Our further observation that hypoxia-responsive genes are not induced in the thymus despite significant cellular hypoxia suggests that adaptation to physiological hypoxia has occurred. This adaptation may alter the balance of pro- and antiapoptotic factors within the thymus, including factors necessary for thymocyte growth and development. Our observations that the magnitude and duration of the change in thymic PO2 varies when 100% O2 is administered (Fig. 3) and that the hypoxia marker pimonidazole continues to accumulate focally in the thymus despite high arterial PO2 (Fig. 1) provide evidence for active regulation of thymic oxygen tension. Further studies are needed to determine the mechanisms by which thymic physiology regulates oxygen tension and gene expression in response to low thymic oxygen tensions.

Our hypoxia marker studies in high-density cultures show that significant differences in pimonidazole adduct formation occur within individual cultures of thymocytes even under normoxic conditions. Hypoxia markers bind predominately to thiol molecules, producing acid soluble glutathione and cysteine adducts as well as the acid-insoluble protein adducts that are detected immunohistochemically. Rigorous determination of the cell cycle dependence of soluble thiols in thymocytes will be required to fully understand variations in pimonidazole binding among thymocytes at different stages of the cell cycle. However, when the pimonidazole reactivity of thymocytes is considered at a given stage of the cell cycle, it is clear that a significant number of cells in high-density cultures experience PO2 <10 mmHg even when cultured under normal ambient O2 concentrations (~160 mmHg). These results suggest that the PO2 experienced by many cells cultured in the hypoxia chamber may have been much lower than the atmosphere of 1.5% O2, thus predisposing these cells to anoxic death (necrosis). Studies to determine whether these thymocytes would undergo necrosis when cultured in hypoxic gas mixtures using nominal O2 concentrations higher than 1.5% are in progress.

In summary, these studies demonstrate that normal thymus is physiologically hypoxic and that regulatory mechanisms exist to maintain thymic cellular hypoxia in vivo. They further suggest that oxygen tension may regulate thymocyte survival both in vitro and in vivo. A study by Caldwell et al. (6) recently demonstrated that physiologically relevant low oxygen tensions could regulate T cell receptor-triggered lymphokine secretion as well as the development and activity of cytotoxic T lymphocytes. Thus oxygen tension may regulate lymphocyte function at multiple stages during T cell development and immune function.
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