RESEARCH ARTICLE

Experimental Hypoxia and Embryonic Angiogenesis†

O. Naňka,[1]* P. Valášek,[1,2] M. Dvořáková,[3] and M. Grim[1]

We examined the role of hypoxia and HIF factors in embryonic angiogenesis and correlated the degree of hypoxia with the level of HIF and VEGF expression and blood vessel formation. Quail eggs were incubated in normoxic and hypoxic (16% O_2) conditions. Tissue hypoxia marker, pimonidazol hydrochloride, was applied in vivo for 1 hr and detected in sections with Hypoxyprobe-1 Ab. VEGF and HIF expression was detected by in situ hybridization. HIF-1α protein was detected in sections and by Western blot. Endothelial cells were visualized with QH-1 antibody. Hypoxic regions were detected even in normoxic control embryos, mainly in brain, neural tube, branchial arches, limb primordia, and mesonephros. The expression patterns of HIF-1α and HIF-1β factors followed, in general, the Hypoxyprobe-1 marked regions. HIF-2α was predominantly expressed in endothelial cells. Diffuse VEGF expression was detected in hypoxic areas of neural tube, myocardium, digestive tube, and most prominently in mesonephros. Growing capillaries were directed to areas of VEGF positivity. Hypoxic regions in hypoxic embryos were larger and stained more intensely. VEGF and HIF-1 factors were proportionately elevated in Hypoxyprobe-1 marked regions without being expressed at new sites and were followed by increased angiogenesis. Our results demonstrate that normal embryonic vascular development involves the HIF-VEGF regulatory cascade. Experimentally increasing the level of hypoxia to a moderate level resulted in over-expression of HIF-1 factors and VEGF followed by an increase in the density of developing vessels. These data indicate that embryonic angiogenesis is responsive to environmental oxygen tension and, therefore, is not entirely genetically controlled. *Developmental Dynamics 235:723–733, 2006.* © 2006 Wiley-Liss, Inc.

Key words: hypoxia; embryo; quail; VEGF; angiogenesis; HIF; pimonidazol; hypoxyprobe

Accepted 16 December 2005

INTRODUCTION

Blood vessels develop during embryogenesis either by vasculogenesis (de novo formation from precursor cells) or by angiogenesis (from pre-existing vessels) (Risau, 1997). These processes are in part controlled by vascular endothelial growth factor (VEGF). It has recently been shown that VEGF exists in five forms designated VEGF A, B, C, D, E. Also three members of the VEGF receptor family, VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1/Quek 1), and VEGFR3 (Flt-4/Quek 2), were identified with unique characteristics in terms of regulation of angiogenesis and vascular permeability (Weinstein, 1999).

Evidence that hypoxia resulting from increased metabolic demands triggers angiogenic factors was first provided by experiments on growing tumors (Folkman et al., 1971). Cells at low oxygen tension express hypoxia-induced transcription factor, HIF-1α (Semenza and Wang, 1992; Forsythe et al., 1996). Furthermore, this factor is relatively stabilized under hypoxia, as HIF-1α hydroxylase, which starts the degradation pathway, requires oxygen (Brahimi-Horn et al., 2001). Undegraded HIF-1α then translocates to the nucleus where it forms HIF-1

†This article was accepted for publication in *Developmental Dynamics* 235#1–Cardiovascular Special Issue
[1]Institute of Anatomy, Charles University First Faculty of Medicine, Prague, Czech Republic
[2]Royal Veterinary College, London, United Kingdom
[3]Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic
Grant sponsor: Ministry of Education of The Czech Republic; Grant numbers: VZ 1111 00003-3 and 0021620806.
***Correspondence to: Ondrej Nanka, Institute of Anatomy, Charles University First Faculty of Medicine, U nemocnice 3, CZ-128 00 Prague 2, Czech Republic. E-mail: ondrej.nanka@lf1.cuni.cz**

DOI 10.1002/dvdy.20689
Published online 27 January 2006 in Wiley InterScience (www.interscience.wiley.com).

transcription factor by dimerising with HIF-1β (ARNT) that is expressed constitutively. One of the first described angiogenic factors that are regulated by HIF-1 is VEGF (Shweiki et al., 1992). HIF-1 not only activates VEGF expression but also other genes that increase O_2 delivery such as erythropoetin and glycolytic enzymes (reviewed by Blancher and Harris, 1998). HIF-2α is also induced by hypoxia and again together with HIF-1β binds the regulatory regions of hypoxia responsive genes (Tian et al., 1997; Kappel et al., 1999; Ema et al., 1997).

The measurements of oxygen tension in organ primordia of 4- and 6-day-old chick embryos have shown very low values. Using oxygen microelectrodes, Meuer and Baumann (1987) and Meuer et al. (1992) showed that tissue pO_2 values ranged between zero and 5.9 kPa in arterial blood. About half of the obtained values were lower than 0.65 kPa. The oxygen tension in brain was 2 and 0.7 kPa in wing and limb buds (Meuer et al., 1992). Areas of tissue hypoxia in fast-growing organ primordia thus could represent a physiological stimulation of angiogenesis via HIF-induced VEGF. Immunohistochemical detection of tissue hypoxia using pimonidazole (Raleigh et al., 1998) reveals hypoxic regions and allows for correlation with the level of expression of genes participating in this cascade. Pimonidazol forms immunologically detectable adducts only in tissues with partial oxygen tension below 1.3 kPa (10 mm Hg). The critical pO_2 for efficient oxidative metabolism in mitochondria is 0.133 kPa of O_2 (Chance et al., 1962; Chance and Williams, 1955). Currently, there are only a few studies demonstrating hypoxic regions in embryonic organ primordia using bioreductive markers of hypoxia. Lee et al. (2001) used pimonidazol for analysis of the role of tissue hypoxia in mouse embryos. Catron et al. (2001) described an increase in covalent binding of 3H-misonidazole in chick heart (ED9) exposed to chronic hypoxia and Sugishita et al. (2004) used nitroimidazol EF5 for detection of hypoxic region of the embryonic cardiac outflow tract in chick and quail.

Lee et al. (2001) used normal mouse embryos to analyze the hypoxia as a possible signal for vessel development. We decided to extend this type of study using experimental hypoxia in a model of avian embryos. Our study includes the detection of all HIF factors and deploys the QH1 antibody as quail endothelia specific marker. Quail eggs were incubated not only under normal condition but also at lower pO_2 tensions to test to what extent the hypoxia could be efficient in stimulation of angiogenesis. Our data show that areas of tissue hypoxia in fast-growing embryonic organ primordia provide a milieu for physiological stimulation of angiogenesis.

RESULTS

Survival of Hypoxic Embryos and Macroscopic Findings

Preliminary experiments with hypoxia from the beginning of incubation always resulted in non-viable embryos at 48 hr. Our further experiments therefore started the hypoxic conditions from ED2 onwards. On ED4, after 2 days of hypoxia, the majority of embryos was viable and on ED6 (after 4 days of hypoxia) approximately 2/3 were viable. The longest period of survival was 9 days. The embryos that did not survive showed diffuse subcutaneous bleeding but no macroscopic malformations. Embryos incubated under hypoxic conditions were usually larger than the controls. The size difference was more prominent at ED6 than at ED4, however the individual variability was great. By determining the total protein content in some embryos it was verified, that the weight elevation was caused by edema.

Hypoxic Regions in Normoxic Embryos

Hypoxyprobe 1 detects the tissue hypoxia when pO_2 is less than 1.3 kPa (10 mm Hg) (Raleigh et al., 1998). Low diffuse staining intensity was observed in all tissues of the body (Fig. 1A). The higher intensity of staining was detected in some organ primordia including lungs, liver, neural tube, mesonephros, myocardium, and in branchial arches at ED4 (Fig. 1F). Limb primordia at ED4 exhibited hypoxia in subectodermal layer of mesenchyme, especially under the apical ectodermal ridge (Fig. 2G).

ED6 embryos displayed hypoxic areas within the whole extent of lung and liver, in gray matter of the neural tube, in proximal tubules and glomeruli of mesonephros, in Wolffian duct, in septal myocardium, in mucous and muscular layers of digestive tube, and in muscle primordia of limbs (Fig. 3I,M,Q).

Hypoxic Regions in Hypoxic Embryos

The staining intensity of Hypoxyprobe reaction in hypoxic conditions was much higher than in normoxic condition. The hypoxic areas became larger and were observed everywhere where organ primordia were formed by densely packed cells (Fig. 1B). They were more pronounced in ED6 than in ED4 embryos (Figs. 2E, 3K,O,S). In limb primordia of ED6 embryos, hypoxic regions included perichondrium and primordia of tendons and muscles (Figs. 2I, 3W).

Expression of HIF Factors at ED 4 in Normoxia

The HIF-1α and HIF-1β mRNA were expressed under normoxic conditions at high levels in numerous localizations, including the neuroepithelium of the brain vesicles, in the subependymal layer of the neural tube, in ventral parts of dermomyotomes, and in auditory vesicle, inner retina layer, branchial arches 1 and 2 (Fig. 1G), limb primordia, and cloacal region. The signal was weak and diffusely distributed in limb primordia (Fig. 1C,O).

HIF-2α expression was found only in the endocardium and endothelium of blood vessels: in the dorsal aorta, arteries of branchial arches, intersomitic vessels (Fig. 1Q), and vessels of mesonephros.

Detection of HIF-1α protein in sections showed weak activity in normoxic embryos, especially in myocardium, liver, and mesonephros (Fig. 1I).

Expression of HIF Factors at ED 4 in Hypoxia

The HIF-1α and HIF-1β mRNA expression under hypoxic conditions ex-

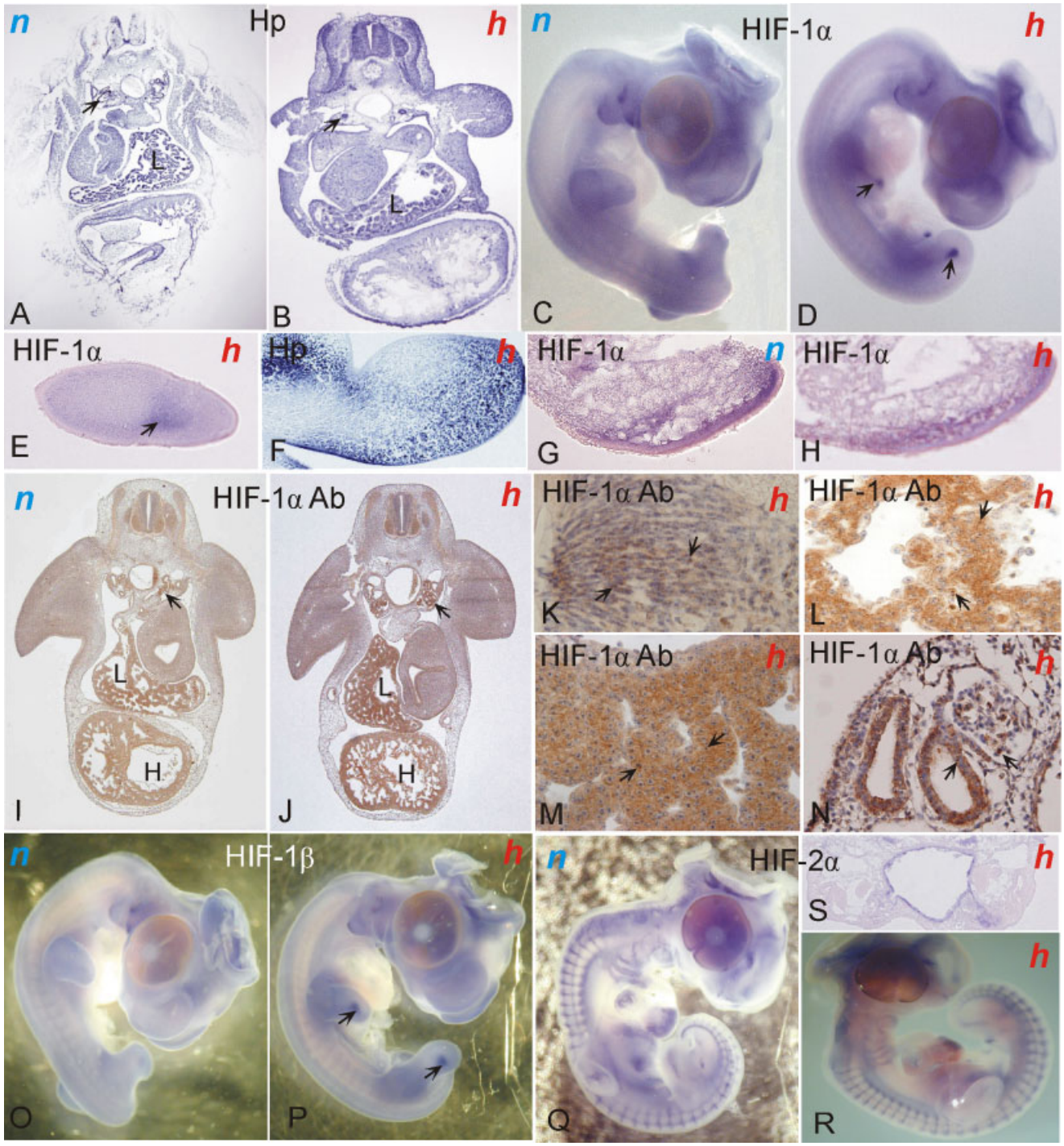

Fig. 1. Hypoxia and HIF gene expression at ED4 in normoxic (*n*) and hypoxic (*h*) embryos. **A,B:** Hypoxic regions detected with Hypoxyprobe-1 on transverse sections of normoxic (A) and hypoxic embryos (B). Blue reaction product is visible in proximal tubuli of mesonephros (arrows), liver primordia (L), developing heart, neural tube, and limb buds. Note the stronger intensity of reaction in hypoxic embryo. **C,D:** HIF-1α mRNA expression in normoxic (C) and hypoxic (D) embryo. Strong signal in branchial arches, weaker signal in limb and in brain. Note distinct localisation in limb primordia of hypoxic embryo (arrows). **E:** Expression of HIF-1α mRNA in axial structures of a limb bud in a hypoxic embryo (section from D). **F–H:** Sections of branchial arches. Detection of tissue hypoxia in hypoxic embryo (F). HIF-1α mRNA expression in normoxic (G) and hypoxic (H) embryo. Counterstaining with nuclear red (G, H). **I,J:** Immunohistochemistry of HIF-1α protein expression in transverse section of normoxic (I) and hypoxic (J) embryo. The staining intensity is stronger in tissues of hypoxic embryo (J). Note stronger intensity of reaction in liver (L), heart (H), and proximal tubuli of mesonephros (arrow). Counterstaining with hematoxylin. **K–N:** Details of organs from hypoxic embryo (J), neural tube (K), myocardium (L), liver (M), and mesonephros (N). The protein is localized in cytoplasm and in nuclei (arrows). **O,P:** HIF-1β mRNA expression in normoxic (O) and hypoxic (P) embryo. Note distinct localisation and stronger positivity in limb primordia of hypoxic embryo (arrows). **Q,R:** Expression of HIF-2α mRNA in endothelial cells of normoxic (Q) and hypoxic (R) embryo. Note dorsal aorta, intersomitic vessels, vessels of the branchial arches, subcutaneous plexuses, and endocardium. **S:** Transverse section of whole mount HIF-2α mRNA in situ hybridized embryo shows signal in aortic endothelium. Counterstaining with nuclear red.

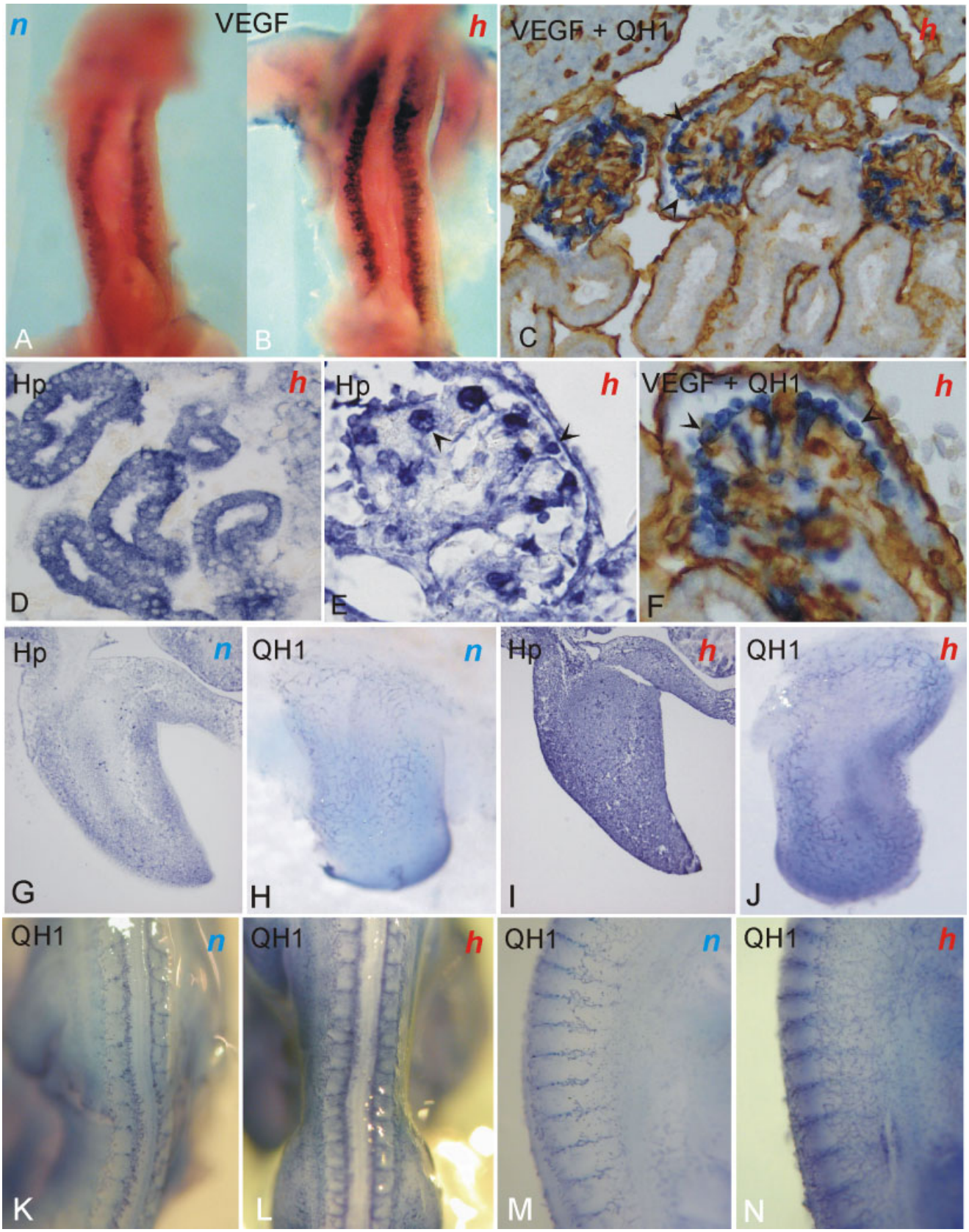

Fig. 2. VEGF expression, detection of hypoxia, and visualization of capillaries in normoxic (*n*) and hypoxic (*h*) embryos at ED4 and ED6 (C–F). **A,B:** Ventral views of dissected mesonephros. ISH for VEGF mRNA in normoxic (A) and hypoxic (B) embryos. Note increased VEGF expression under hypoxic conditions. **C–F:** Sections of mesonephros at ED 6. VEGF (blue) is expressed in podocytes (C, F) (arrowheads) but not in tubules. Numerous simultaneously immunostained glomerular capillaries (brown) are likely to be stimulated in growth by VEGF from podocytes (C, F). The Hypoxyprobe-1 detects hypoxia in proximal tubules (D) and in podocytes (arrowheads) (E). **G–J:** Sections of limb primordia. Hypoxic regions (G, I) and visualization of capillaries by QH1-Ab (H, J) in the developing wing in normoxic (G, H) and hypoxic (I, J) embryos. Hypoxia is more pronounced in developing marginal sinus. **K–N:** Subcutaneous intersomitic vessels stained with QH-1 antibody on dorsal (K, L) and lateral (M, N) aspects of ED 4 normoxic (K, M) and hypoxic (L, N) embryos. Vascular plexuses are richer in hypoxic embryos.

hibited similar localization as in normoxic embryos (Fig. 1C,D,O,P). However, the intensity of expression of both factors was higher and areas of positivity were larger. These differences were pronounced in limb primordia, mainly in the subectodermal mesenchyme and in a strip in the axis of zeugopodial limb segment (Fig. 1E). The strip ends distally by a dot of positivity in the autopodial segment (Fig. 1D,P). This strip corresponds to the position of the main arterial trunk at ED6. There were no differences in expression of HIF-2α in endothelium and endocardium under hypoxic and normoxic conditions (Fig. 1Q–S).

The visualization of HIF-1α protein in sections (Fig. 1J) has shown an increased activity especially in neural tube (Fig. 1K), myocardium (Fig. 1L), liver (Fig. 1M), mesonephros (Fig. 1N), and in muscle primordia. The reaction product was localized in cytoplasm and in nuclei. Western blot analysis suggests an approximately 2–3 times higher amount of HIF-1α protein in hypoxic embryos (Fig. 4). This observation is consistent with known HIF-1α stabilization due to increased hypoxia.

Expression of HIF Factors at ED 6 in Normoxia

The highest level of HIF-1α and HIF-1β mRNA expression at ED6 was observed in brain vesicles, especially in mesencephalon, in the subependymal zone of neural tube, in otic vesicle, and in the mandibular process. The level of HIF-1α and β expression in the heart is low. The signal in the trunk is visible in the dorsal and ventral parts of myotomes. The strip of expression of HIF-1α and β, visible in the axis of zeugopodium, has at ED6 shifted distally in rays of digit primordia (Fig. 3C,E). Areas of high staining intensity in stylopodium and zeugopodium correspond to muscle primordia (data not shown). The HIF-2α expression in the endothelium of blood vessels remained unchanged (Fig. 3G).

Expression of HIF Factors at ED 6 in Hypoxia

The expression of HIF-1 factors mRNA was elevated under hypoxic conditions when compared with normoxia. It remained restricted to their original sites, without expression at new sites (Fig. 3D,F).

The increased overall HIF-2α expression correlated to the increased vascular density. However, the expression remained localized to the endothelium of blood vessels and was of the same intensity as in normoxia (Fig. 3H).

VEGF Expression and Angiogenesis in Hypoxic Regions

Mesonephros.

VEGF expression was found in mesonephros primordium at ED4 (Fig. 2A,B) and more specifically in podocytes at ED6. This expression was stronger in hypoxia and was in both instances accompanied with QH1-positive endothelia (Fig. 2C,F). In addition, weak VEGF expression was visible in proximal tubules in hypoxia and the tubules were also positive for Hypoxyprobe (Fig. 2D). They were surrounded by rich capillary plexuses in sections simultaneously stained with QH1 Ab (Fig. 2C). This arrangement in mesonephros thus suggests that growth of capillaries in mesonephros is stimulated by hypoxia and VEGF. This was most obvious on the glomerular capillaries where hypoxic podocytes produce VEGF (Fig. 2E,F).

Neural tube.

ISH of the neural tube was positive for VEGF expression in the whole extent of the gray matter in normoxic ED4 and ED6 embryos. The spinal cord was surrounded by a capillary plexus. In hypoxic embryos, the VEGF expression was more intense in anterior horns and in basal plate than in the remaining parts of the tube. The basal plate area appeared to be the site of entry of vessels in the neural tube (Fig. 3I–L).

Digestive tract.

Cross-sections of the intestine showed distinct differences in the level of staining with Hypoxypobe and VEGF expression between normoxic and hypoxic embryos. In contrast to normoxic embryos, the mucous and muscular layer of intestine was strongly positive in both stains under hypoxia.

The vascularization of the intestinal wall at ED6 of hypoxic embryos was markedly more developed than in the normoxic embryos. Hypoxia thus induced acceleration of angiogenesis in the wall of the intestine. A similar effect of the hypoxic condition was observed in the liver primordium (Figs. 1A,B, 3M–P).

Heart.

Marked differences between normoxic and hypoxic myocardium were observed at ED6. Hypoxia caused excessive trabecularization of ventricular myocardium. Hypoxic regions were visible in septal and superficial (epicardial side) parts of myocardium and stained more intensely under hypoxia. A much higher level of VEGF expression was visible in the whole extent of myocardium in hypoxic embryos than in the normoxic ones (Fig. 3Q–T). This effect was especially pronounced in the interventricular septum, where increased VEGF expression was followed by higher vasularization (Fig. 3U,V).

Limbs.

VEGF expression at ED4 and ED6 was diffuse and low. Under hypoxic conditions, the VEGF was elevated at both ED4 and ED6. At ED6, the high VEGF expression was visible in muscle and tendon primordia and in cartilage forming the skeleton primordia. Capillary plexuses formed around these primordia (Fig. 3X,Y).

Superficial capillary plexuses.

Comparison of the density of subepidermal capillary plexuses in normoxic and hypoxic embryos in whole mounts stained with QH1 antibody allows for the evaluation of the extent of more intense angiogenesis caused by hypoxia.

The intersomitic capillary plexuses on the dorsal and lateral aspects of the trunk and on the dorsal aspects of developing limbs (Fig. 2J,L,N) were more dense in hypoxic embryos, than in normoxic ones (Fig. 2H,K,M).

DISCUSSION

Our results demonstrated the extent of embryonic tissue hypoxia under normal conditions during develop-

ment in liver, mesonephros, neural tube, heart, and limb primordia. Under hypoxic conditions, the staining intensity of hypoxic regions increased and the areas of staining became broader. There was a temporal and spatial correlation between the tissue hypoxia (Fig. 3A,B) and the increased expression of HIF-1 factors followed by VEGF expression and increased angiogenesis. The newly formed vessels were oriented to the regions of increased VEGF expression. Expression of HIF-2α reflected the increased vascular density but remained of the same intensity. Generally, we have shown the effect of hypoxia on the initiation of signaling cascade: increased HIF-1α mRNA → stabilization of HIF-1α protein by hypoxia → VEGF → angiogenesis. It was markedly pronounced in the intestinal wall, mesonephros, neural tube, and developing limbs.

Markers of Hypoxia

A method for determination of tissue oxygenation that has found wide acceptance is polarographic O_2 Eppendorf histography (Meuer et al., 1992; Meuer and Baumann, 1987; Vaupel et al., 1989). Its invasive nature limits the measurement to easily accessible tissues. An alternative approach is the measurement of tissue hypoxia. Nitroimidazole derivatives EF5 (Evans et al., 1995) and pimonidazole (Raleigh et al., 1998) form immunologically detectable adducts in hypoxic tissues. Pimonidazole, in contrast to EF5, has the benefit of prolonged lifetime of the adduct and low toxicity. The use of pimonidazol has been validated in both tumor and normal tissues (Raleigh et al., 1998; Lee et al., 2001). Limitation of the use of injected substances is that they do not diffuse sufficiently in tissues that are not vascularised. This is, however, not an issue in young embryonic stages used in our experiments. We were, thus, able to document the matching of hypoxic regions with HIF-1α expression pattern.

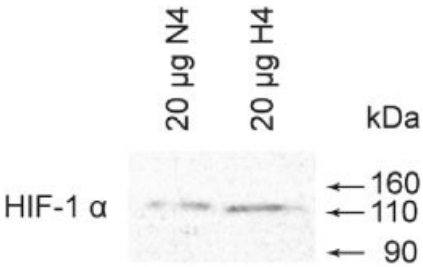

Fig. 4. Immunoblot of HIF-1α protein from whole normoxic and hypoxic quail embryo at ED 4.

Fatal Initial Hypoxia, Edema, VEGF, and Blood Vessel Permeability

Our preliminary experiments showed that the oxygen tension of 16 kPa (≅ 16% O_2) from the beginning of incubation is lethal for all quail embryos. In contrast, the hypoxia permitted the development of the majority of embryos when introduced from the 3rd day of incubation, i.e., from the day when the area vasculosa starts to function as the respiratory organ (Cirotto and Arangi, 1989).

The regimen of introducing hypoxia on the 3rd day of development has also been used by others (Adair et al., 1987, 1988; Mancini et al., 1991; Burton and Palmer 1992).

Edema with the resulting increase of weight is likely explained by increased expression of VEGF. This growth factor was originally called vascular permeability factor according to its first observed effect (Senger et al., 1983). It is worth noting that chick embryos in our preliminary experiments were even more susceptible to the hypoxic conditions than the quail embryos, and we did not find any period of 16 kPa hypoxia, which would permit their embryonic development.

Physiological Embryonic Tissue Hypoxia and the Effect of Hypoxic Conditions

Rapid growth of embryonic tissue and the resulting intensity of metabolic processes represent enhanced demands for oxygen supply. Hypoxic regions observed in our study in quail embryos (neural tube, myocardium, mesonephros, liver, digestive tube) correspond to those observed in normoxic mouse embryos (Lee et al., 2001). In addition, we found hypoxic regions in branchial arches and limb primordia that were not analyzed in mouse by Lee et al. (2001).

Previous results have shown that HIF-1α mRNA and protein expression are tightly coupled to O_2 concentration in different organs of adult rats or mice (Wiener et al., 1996; Yu et al., 1998). The oxygen tension in embryonic organ primordia is much lower than in adults (Meuer et al., 1987, 1992). It is, therefore, not surprising

Fig. 3. Tissue hypoxia, HIF, and VEGF expression in normoxic (*n*) and hypoxic (*h*) embryos at ED 6. **A, B:** Expression of VEGF mRNA is higher in hypoxic embryo (B) than in embryo incubated at normoxia (A). **C, D:** Expression of HIF-1α mRNA in normoxic (C) and hypoxic (D) embryo. Expression is higher in hypoxic embryo. **E, F:** Expression of HIF-1β mRNA in normoxic (E) and hypoxic (F) embryo. Expression is higher and areas of positivity are larger in hypoxic embryo. **G, H:** Expression of HIF-2α mRNA in endothelium of blood capilaries of normoxic (G) and hypoxic (H) embryos. The development of capillary plexuses is more advanced in hypoxic embryo. **I–L:** Transverse sections of the neural tube stained with Hypoxyprobe (I,K) or double stained with QH-1 Ab (brown) and by ISH for VEGF mRNA (blue) (J,L). Tissue hypoxia is more pronounced in grey matter of hypoxic embryo (K) than in the control (I). Correspondingly, in hypoxic embryo (L), the VEGF expression is higher and capillary plexuses are richer than in normoxia (J). **M–P:** Transverse sections of digestive tube show discrete rings of tissue hypoxia (M, O) and corresponding VEGF expression (N, P) in the mucous membrane and the muscular layer of the tube. Note their increased levels (O, P) and richer capillaries (P) in hypoxic embryo in comparison with control (M, N). Some capillaries start to grow into the muscular layer (P). **Q–T:** Section of heart ventricles stained with Hypoxyprobe (Q, S) or with QH-1 Ab (brown) and by ISH for VEGF (R, T). Tissue hypoxia is detected in ventricular wall and interventricular septum of normoxic specimen (Q). In hypoxic embryo, these areas are larger and include also trabecular myocardium (S). VEGF expression is elevated in hypoxic myocardium (T). Note richer trabecularization of myocardium in hypoxic embryo (S, T). **U, V:** Details (from R, T) of interventricular septum. Note the increased vascularization in hypoxic septum (V) in comparison to normoxic one (U). **W–Y:** Longitudinal (W) and transverse (X, Y) sections of limbs stained with Hypoxyprobe (W) or with QH-1 Ab (brown) and by ISH for VEGF (X, Y). In hypoxic limbs, the tissue hypoxia (W) and VEGF expression (Y) are detected in primordia of muscles (m), tendons, and cartilages (c) that are surrounded by capillary plexuses. In normoxic embryo (X), the level of VEGF expression is lower. Muscle primordia are still avascular at ED6.

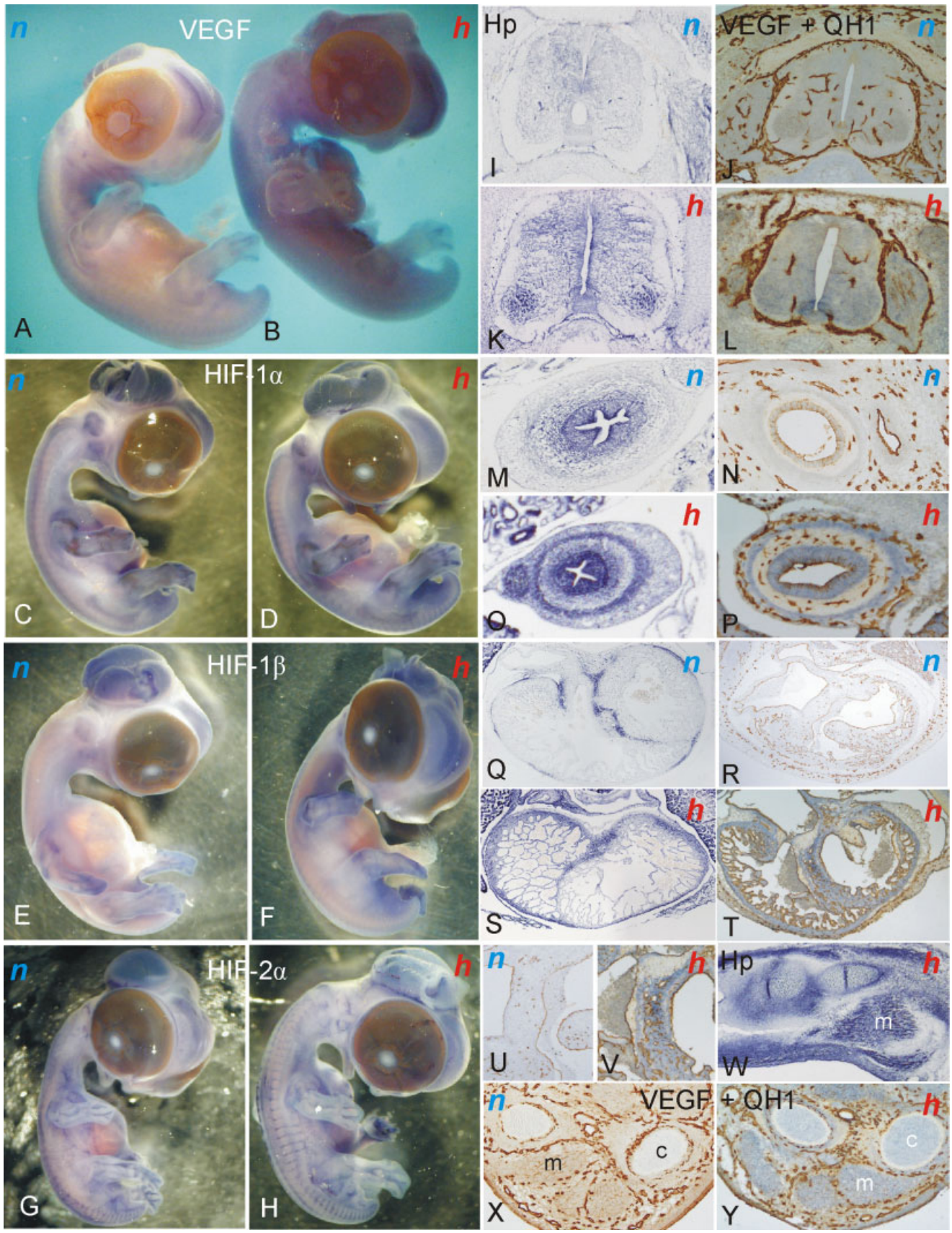
n
VEGF
h
A
B
Hp
n
I
VEGF + QH1
n
J
h
K
h
L
n
HIF-1α
h
C
D
n
M
n
N
h
O
h
P
n
HIF-1β
h
E
F
n
Q
n
R
h
S
h
T
n
HIF-2α
h
G
H
n
U
h
V
Hp
h
m
W
n
VEGF
+ QH1
h
m
c
X
c
m
Y

Fig. 3.

that the basal levels of HIF-1α mRNA and protein were relatively high. Their expression increased under experimental induced hypoxia. The observed increase of HIF-1α protein under hypoxic conditions is likely to be a result of both its increased mRNA levels and its stabilization due to the decreased activity of HIF-1α hydroxylase (Brahimi-Horn et al., 2001).

All hypoxic regions are characterized by expression of HIF-1α mRNA and protein and by VEGF expression that is followed by vessel formation. It has been previously shown that expression of VEGF induced by hypoxia is instrumental in vascularization of the developing kidney in vitro in rat metanephric organ cultures (Tufro-McReddie et al., 1997). Glomerular podocytes are particularly rich sources of VEGF (Freeburg et al., 2003). Our experimental hypoxia led to a strong elevation of VEGF expression that was not observed to such an extent in any other organ primordia. Thus, mesonephros appears to be the most sensitive embryonic organ with respect to VEGF production in reaction to a hypoxic environment.

Quail embryos demonstrated elevated expression of both HIF-1α and HIF-1β under hypoxic conditions. In contrast, the expression of HIF-1β in hypoxic mouse embryos was not influenced by hypoxia (Wang et al., 1995). Whether this represents a difference between in ovo and in utero development remains to be determined.

The observed hypoxic regions with distinct HIF expression were the same regions that were defective in the mouse HIF-1α null mutant. These included hypoplastic or absent vessels in the head, in the branchial arches, and along the dorsal aortae. Intersomitic vessels irregularities were also observed (Iyer et al. 1997; Kotch et al., 1999). The dilatation and regression of vessels in HIF-1α null mutant mouse was caused by extensive cell death of mesenchymal cells. These defects were less severe than in the case of VEGF null mutants (Carmeliet et al., 1996; Ferrara et al., 1996), but it documented that HIF-1α itself participated not only in metabolic cellular adaptations but also in vessel morphogenesis because levels of VEGF were normal or elevated in this mutant (Kotch et al., 1999).

In our experiment, the increased VEGF expression correlated with increased HIF-1α. HIF-1α is known to activate the transcription of VEGF and other genes increasing O_2 delivery and glycolytic enzymes (reviewed by Blancher and Harris, 1998). However, Kotch et al. (1999) found in HIF-1α null mutant that the VEGF was directly induced by hypoxia or glucose deprivation, suggesting there are also other pathways independent of HIF-1α signaling.

Myocardium, Hypoxia, and Angiogenesis

In hypoxic embryos, the wall of the ventricular myocardium was thinner than in normoxia and was positive in the Hypoxyprobe reaction in whole extent. This was in contrast to normoxic embryos where only the septum and the superficial layer of the myocardium were positive. The extensive tissue hypoxia was followed by over-expression of VEGF in myocardium. The most conspicuous feature of the hypoxic heart was the extensive trabecularization of ventricular myocardium. It could represent an adaptive mechanism, shortening the diffusion distance of oxygen for the myocardium.

Our finding of a high level of VEGF expression in the interventricular septum correlates with the observation of Tomanek et al. (1999) in the normoxic heart of the quail where it was followed by a high density of vascularization. The overproduction of trabeculae and increased endocardial cell proliferation were also observed in quail embryos after application of VEGF (Feucht et al., 1997) and in mouse embryos with an elevated expression of VEGF (Miquerol et al., 2000). However, the increased level of VEGF in these experiments resulted in cardiovascular malformations and embryonic lethality. It appears that an adequate level of VEGF expression is important for normal heart morphogenesis.

Another role of hypoxia in developing avian heart has been demonstrated by Sugishita et al. (2004). These authors have shown that regional myocardial hypoxia leads to apoptosis of cardiomyocytes, which triggers remodeling of the cardiac outflow tract.

The Effect of Enhanced Hypoxia

In the hypoxic condition, regions of tissue hypoxia were more pronounced and enlarged. It was accompanied by more extensive HIF-1 and VEGF expression and by a higher density of capillary plexuses.

Superficial capillary plexuses were richer in limbs and in the trunk of embryos incubated under hypoxia than in normoxic conditions. Cross-sections of extremities of hypoxic embryos showed more intensive VEGF expression in muscle primordia and in cartilaginous skeletal primordia. The surrounding capillary plexuses were of richer density than in normoxic embryos. The richer capillary plexuses around the neural tube in hypoxic embryos correlated with stronger VEGF expression in the neural tube. These findings demonstrate that enhanced moderate hypoxia accelerates and intensifies the signal cascade: hypoxia: → HIF-1 → VEGF → vessel formation, and it supports the notion that HIF-1α regulates the formation of capillary networks rather than formation of larger vessels (Ryan et al., 1988).

In the limb primordia, the HIF-1α and β expression was localized in a strip in the axis of the zeugopodial limb segment that was not visible in normoxic embryos. The strip ended distally by a dot of positivity in the autopodial segment. This corresponds to the course of the developing axial arterial trunk and suggests that the position of this artery is determined by local hypoxia and HIF expression. In this case, the local microenvironment seems to be a factor that influences the formation not only of capillary plexuses but of the large vessels as well.

In conclusion, we have presented evidence that small regions of tissue hypoxia are likely to affect the angiogenic response in quail embryos at embryonic days 4 to 6. Factors that activate VEGF expression and the formation of capillary networks are expressed in hypoxic regions. These results document that local tissue hypoxia is a part of the physiological mechanism regulating embryonic vessel formation.

Experimentally enhanced hypoxia

resulted in increased levels of HIF and VEGF followed by increased density of developing vessels. This demonstrates that developmental angiogenesis could be influenced by the microenvironment and is, thus, not entirely determined by genes. Experimentally enhanced hypoxia in avian embryos represents an amenable model for the study of the role of hypoxia in embryonic angiogenesis. It is technically easy and is avoiding any influences from the maternal organism.

EXPERIMENTAL PROCEDURES

The effect of hypoxia on embryonic angiogenesis was studied in embryos of Japanese quail (*Coturnix coturnix japonica*) at embryonic day 4 (ED 4) and 6 (ED 6), i.e., at stages HH 22–24 and 28–30 (Hamburger and Hamilton, 1951). Fertilized eggs were incubated at 38°C and 60–85% humidity under normoxic (21 kPa) conditions for the first 48 hr and subsequently for 48 or 96 hr under hypoxic (16%O_2 ≅ 16 kPa O_2) conditions. Controls were incubated in normoxic atmospheric oxygen tension.

Western Blot Analysis

The tissue of normoxic and hypoxic embryos were dissolved in 0.5 % SDS, 50 mM TrisHCl, pH 6.8, and total proteins were determined. Protein concentrations were adjusted to 2 μg/1 μl and 5× sample buffer was added. Denatured lysates (100°C, 5 min) were separated on 8% polyacrylamide gel and proteins were transferred onto Hybond-ECL membrane (Amersham).

Membranes were blocked with 5% nonfat dry milk for 45 min, incubated 1 hr with the rabbit polyclonal antibody (Abcam No. 16535) recognizing the HIF-1α (1:500 in PBS + 0.5% BSA) at 25°C, washed 3× in TBST (10 mM TrisHCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20), and exposed to secondary anti-rabbit antibody (Amersham, NA934) 1:10,000 (in PBS+0.5% BSA) for 1 hr at 25°C. Membranes were then washed 5× in TBST, developed with ECL reagents (Amersham) and exposed to X-ray film.

Staining With QH-1 Ab in Paraffin Sections

Embryos were collected at appropriate stages, weighed without embryonic membranes, fixed in 4% PFA/PBS, and prepared for Paraplast embedding and staining with QH1 antibody (Hybridoma Bank, University of Iowa). This antibody stains quail vascular endothelial cells and haemopoietic cells (Pardanaud et al., 1987). Paraffin sections were preblocked in 5% goat serum in PBS, and then incubated 90 min with QH1-Ab (1:1,000) at room temperature. Endogenous peroxidase was quenched by 0.1% H_2O_2 in methanol for 20 min. Secondary antibody (Goat anti mouse Px; Sigma: A4416, diluted 1:200) was applied for 90 min at RT and staining revealed by the DAB color reaction.

Staining With QH-1 on Whole Mount Embryos

Embryos were fixed in 4% PFA/PBS/0.1%Triton, dehydrated in methanol and stored at −20°C. After rehydration, they were treated with Proteinase K (20 μg/ml) for 1 min per each embryonic HH stage at room temperature and post-fixed in 4% PFA/0.25%GA/PBS/0.1%Triton. QH1 Ab was diluted 1:1,000 and embryos were incubated overnight at 4°C on rotating wheel. Extensive hourly washes in PBS/0.5%Triton were followed by overnight incubation with secondary Goat Anti mouse AP (DAKO D0486, 1:500) antibody in PBS/0.5%Triton at 4°C. NBT/BCIP was used as a substrate. Colored embryos were stored at 4% PFA.

Staining With HIF-1α Antibody in Paraffin Sections

HIF-1α rabbit polyclonal antibody (ABCAM 16535) is predicted to react with chick and quail due to sequence homology (90% identity with immunogen). Paraffin sections were preblocked in 5% goat serum in PBS, and then incubated with primary antibody overnight (1:200) at 4°C. Endogenous peroxidase was quenched by 0.1% H_2O_2 in methanol for 20 min. Secondary antibody (Goat anti-rabbit-biotin; Jackson 111-065-144, diluted 1:600) was applied for 60 min at RT and staining revealed by Extravidin/Px followed by DAB color reaction.

ISH on Whole Mount Embryos

In situ hybridizations with HIF-1α, HIF-1β, HIF-2α, and VEGF mRNA probes were performed according to Nieto et al. (1996). Hybridization was performed with digoxigenin labeled antisense riboprobes at 70°C over 1–2 days. Quail HIF-2α 549 bp product of PCR from cDNA of HH15 quail embryos was cloned into pCR 2.1 vector (Invitrogen). Primers ATATGGAC-CAAA- CGGAATCG and CTGTCT-TCATAG- AGGAGGTT were designed according to quail HIF-2α mRNA (Elvert et al., 1999). The other probes were kindly provided by I. Flamme (quail VEGF, 310-bp probe; Flamme et al., 1995), T. Takahashi (chick HIF-1α, 761 bp, GenBank: AB013746; Takahashi et al., 2001), M. K. Walker (chick HIF-1β, 584 bp, GenBank: AF348088; Catron et al., 2001). Fab fragments of sheep antibody to digoxigenin conjugated to alkaline phosphatase mediated the visualization (1:5,000, Roche). Expression patterns of the probes in both chick and quail embryos were identical allowing for their use in quail species.

ISH in Paraffin Sections

The sense and antisense mRNA probes were prepared from quail VEGF cDNA (Flamme et al.,1995; 310 bp) and labeled with digoxigenin RNA labeling kit as recommended by the vendor (Roche). In situ hybridization was carried out according to Nieto et al. (1996). Briefly: embryos were fixed overnight at 4°C in Serra′s fluid (Serra, 1946), dehydrated, embedded in Paraplast, sectioned (14 μm), and mounted onto silanised slides. The sections were treated with proteinase K and post-fixed in 4% PFA. Hybridization temperature was 65°C overnight.

After standard washing, the digoxigenin was detected with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche) in a blocking agent (1% bovine serum albumin in maleate buffer) at 4°C overnight. The antibody was visualized by BCIP/NBT color reaction (Roche) in NTMT buffer for 3–5 days, revealing a blue product. Selected slides were additionally immunolabeled with QH1 antibody.

Detection of Tissue Hypoxia

Hypoxyprobe 1 (pimonidazol hydrochloride, Natural Pharmacia International, Inc., Research Triangle Park, NC) has been used according to recommendations of the producer. After intravenous administration, it is evenly distributed throughout the embryo. Hypoxyprobe-1 was injected in the vitelline (chorioallantoic) vein at ED 4 or 6 (1 μg/1 μl and 18 μg/5 μl in PBS respectively). Embryos were re-incubated for 1 hr and fixed in 4% PFA/PBS. The reaction product was detected on paraffin sections with Hypoxyprobe-1 monoclonal antibody (1:50) for 40 min at room temperature and the secondary antibody conjugated with alkaline phosphatase (1:300) for 1 hr at RT. The color reaction was visualized by NBT/Xphosphate.

ACKNOWLEDGMENTS

We thank Dr. Ingo Flamme, Dr. Mary K. Walker, and Dr. Toshiyuki Takahashi for providing the VEGF, HIF-1α, and HIF-1β probes, respectively. Dr. Raleigh has kindly provided a test sample of Hypoxyprobe. We also thank Dr. Ketan Patel for help with cloning the HIF-2α probe and Ms. Alena Kautská, Eva Kluzáková, and Markéta Pleschnerová for excellent technical assistance.

REFERENCES

Adair TH, Guyton AC, Montani JP, Lindsay HL, Stanek KA. 1987. Whole body structural vascular adaptation to prolonged hypoxia in chick embryos. Am J Physiol 252:H1228–1234.

Adair TH, Montani JP, Guyton AC. 1988. Effects of intermittent hypoxia on structural vascular adaptation in chick embryos. Am J Physiol 254:H1194–1199.

Blancher Ch, Harris AL. 1998. The molecular basis of the hypoxia response pathway: tumour hypoxia as a therapy target. Cancer Metast Rev 17:187–194.

Brahimi-Horn Ch, Berra E, Pouyssegur J. 2001. Hypoxia: the tumor's gateway to progression along the angiogenic pathway. Trends Cell Biol 11:32–36.

Burton GJ, Palmer ME. 1992. Development of the chick chorioallantoic capillary plexus under normoxic and normobaric hypoxic and hyperoxic conditions: a morphometric study. J Exp Zool 262:291–298.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439.

Catron TF, Mendiola MA, Smith SM, Born JL, Walker MK. 2001. Hypoxia regulation of avian cardiac Arnt and HIF-1α mRNA expression. Biochem Biophys Res Commun 282:602–607.

Chance B, Williams G. 1955. Respiratory enzymes in oxidative phosphorylation. III. The steady state. J Biol Chem 217:409–427.

Chance B, Cohen P, Jobsis F, Schoener B. 1962. Intracellular oxidation-reduction states in vivo. Science 137:499–508.

Cirotto C, Arangi I. 1989. How do avian embryos breathe? Oxygen transport in the blood of early chick embryos. Comp Biochem Physiol A 94:607–613.

Elvert G, Lanz S, Kappel A, Flamme I. 1999. mRNA cloning and expression studies of the quail homologue of HIF-2α. Mech Dev 87:193–197.

Ema M, Taya S, Yokotani N, Sogawa K, Matsuda Y, Fujii-Kuriyama Y. 1997. A novel bHLH–PAS factor with close sequence similarity to hypoxia-inducible factor 1α regulates VEGF expression and is potentially involved in lung and vascular development. Proc Natl Acad Sci 94:4273–4278.

Evans SM, Joiner B, Jenkins WT, Laughlin KM, Lord EM, Koch CJ. 1995. Identification of hypoxia in cells and tissues of epigastric 9L glioma using EF5 [2-(2-nitro-1H-imidazol-1-yl)-*N*- (2,2,3,3,3-pentafluoropropyl) acetamide]. Br J Cancer 72:875–882.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442.

Feucht M, Christ B, Wilting J.. 1997. VEGF induces cardiovascular malformation and embryonic lethality. Am J Pathol 151:1407–1416.

Flamme I, Breier G, Risau W. 1995. Vascular endothelial growth-factor (vegf) and vegf receptor-2(flk-1) are expressed during vasculogenesis and vascular differentiation in the quail embryo. Dev Biol 169:699–712.

Folkman J, Merier E, Abernathy C, Wiliams G. 1971. Isolation of a tumor factor responsible for angiogenesis. J Exp Med 133:257–288.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor-1. Mol Cell Biol 16:4604–4613.

Freeburg PB, Robert B, St John PL, Abrahamson DR. 2003. Podocyte expression of hypoxia-inducible factor (HIF)-1 and HIF-2 during glomerular development. J Am Soc Nephrol 14:927–938.

Hamburger V, Hamilton HL. 1951. A series of normal stages in development of the chick embryo. J Morphol 88:49–92.

Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL. 1998. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1α. Genes Dev 12:149–162.

Kappel A, Ronicke V, Damert A, Flamme I, Risau W, Breier G. 1999. Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice. Blood 93:4284–4292

Kotch LE, Iyer NV, Laughner E, Semenza GL. 1999. Defective vascularization of HIF-1α-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Dev Biol 209:254–267.

Lee YM, Jeong CH, Koo SY, Son MJ, Song HS, Bae SK, Raleigh JA, Chung HY, Yoo MA, Kim KW. 2001. Determination of hypoxia region by hypoxia marker in developing mouse embryos in vivo: a possible signal for vessel development. Dev Dyn 220:175–186

Mancini L, Bertossi M, Ribatti D, Bartoli F, Nico B, Lozupone E, Roncali L. 1991. The effects of long-term hypoxia on epicardium and myocardium in developing chick embryo hearts. Int J Microcirc Clin Exp 10:359–371.

Meuer HJ, Baumann R. 1987. Oxygen Supply of Early Chick embryo in Normoxia and Hypoxia. J Exp Zool (Suppl) 1:203–207.

Meuer HJ, Hartmann V, Jopp S. 1992. Tissue PO2 and growth rate in early chick embryos. Respir Physiol 90:227–237.

Miquerol L, Langille BL, Nagy A. 2000. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. Development 127:3941–3946.

Nieto MA, Patel K, Wilkinson DG. 1996. In situ hybridization analysis of chick embryos in whole mounts and in tissue sections. Methods Cell Biol 40:219–235.

Pardanaud L, Altmann C, Kitos P, Dieterlen-Lièvre F, Buck C. 1987. Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. Development 100:339–349.

Raleigh JA, Calkins-Adams DP, Rinker LH, Ballenger CA, Weissler MC, Fowler WC Jr, Novotny DB, Varia MA. 1998. Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. Cancer Res 58:3765–3768.

Risau W. 1997. Mechanism of angiogenesis. Nature 386:671–674.

Ryan HE, Lo J, Johnson RS. 1998. HIF-1α is required for solid tumor formation and embryonic vascularization. EMBO J 17:3005–3015.

Semenza GL, Wang GL. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 12:5447–5454.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985.

Serra JA. 1946. Histochemicals tests for protein and amino acids: The characterization of basic protein. Stain Technol 21:5–18.

Shweiki D, Itin A, Soffer D, Keshet E. 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359: 843–845.

Sugishita Y, Leifer DW, Agani F, Watanabe M, Fisher SA. 2004. Hypoxia-responsive signaling regulates the apoptosis-dependent remodeling of the embryonic avian cardiac outflow tract. Dev Biol 273:285–296.

Takahashi T, Sugishita Y, Nojiri T, Shimizu T, Yao A, Kinugawa K, Harada K, Nagai R. 2001. Cloning of hypoxia-inducible factor 1α cDNA from chick embryonic ventricular myocytes. Biochem Biophys Res Commun 281:1057–1062.

Tian H, McKnight SL, Russell DW. 1997. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev 11:72–82.

Tomanek RJ, Ratajska A, Kitten GT, Yue X, Sandra A. 1999. Vascular endothelial growth factor expression coincides with coronary vasculogenesis and angiogenesis. Dev Dyn 215:54–61.

Tufro-McReddie A, Norwood VF, Aylor KW, Botkin SJ, Carey RM, Gomez RA. 1997. Oxygen regulates vascular endothelial growth factor mediated vasculogenesis and tubulogenesis. Dev Biol 183: 139–149.

Vaupel P, Kallinowski F, Okunieff P. 1989. Blood flow, oxygen and nutrient supply, and metabolic environment of human tumours: a review. Cancer Res 49:6449–6465.

Wang GL, Jiang BH, Rue EA, Semenza GL. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA 92:5510–5514.

Weinstein BM. 1999. What guides early embryonic blood vessel formation? Dev Dyn 215:2–11.

Wiener CM, Booth G, Semenza GL. 1996. In vivo expression of mRNAs encoding hypoxia-inducible factor 1. Biochem Biophys Res Commun 225:485–488.

Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K, Semenza GL. 1998. Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am J Physiol 19L: 818–826.