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Immunohistochemical Detection of Haemoglobin Subunit Epsilon (HBE) in the Developing Mouse Placenta

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Abstract

Introduction: Haemoglobin is a widely studied protein due to its important roles in physiology and pathology. Aberrant expression of haemoglobins, including primitive globins, have been reported in various sites and disease states and may have utility in some instances as diagnostic and/or prognostic markers. Despite this, robust detection of haemoglobin epsilon in the placenta during development by immunohistochemistry has not been well documented.

Aim: To evaluate a polyclonal antibody against human haemoglobin subunit epsilon (HBE) by immunohistochemistry during primitive erythropoiesis in the developing mouse placenta.

Methods and results: An immunohistochemistry protocol was developed using a commercially available anti-human haemoglobin subunit epsilon antibody on the mouse placenta at embryonic day 11.5. Strong and specific cytoplasmic staining was observed in primitive erythroid cells within the blood cell islands. By contrast, the placenta endothelium, mesothelium and mesoderm were all immunonegative for epsilon haemoglobin.

Conclusions: An immunohistochemistry protocol for the specific detection of epsilon haemoglobin was successfully developed using mouse placenta tissue. This assay has utility as a tool for the study of erythropoiesis during development and/or detecting the ectopic expression of epsilon globins in disease states such as cancer.

Keywords: DAB staining; Polyclonal antibody; Primitive haemopoiesis; Foetal erythrocytes

Abbreviations: DAB: 3,3’-Diaminobenzidine; FFPE: Formalin Fixed Paraffin Embedded; Hb: Haemoglobin; HBE: Haemoglobin Subunit Epsilon; H&E: Hematoxylin and Eosin; IHC: Immunohistochemistry; TBS: Tris-Buffered Saline

Introduction

Globin is an essential protein involved in a variety of biological functions and distributed in all life kingdoms [1,2]. It can be divided into four major groups: cytoglobin, neuroglobin, myoglobin and haemoglobin (Hb) with different functions and tissue distributions [3]. Beginning in the yolk sac blood islands, Hb in erythroid cells undergoes successive switching during gestation from embryonic to foetal (primitive) and then to adult (definitive) Hb in humans, and directly from embryonic to adult Hb in mice [4]. These haemoglobins are constructed by combining two α-like globins (embryonic ζ or adult α) with two β-like globins (embryonic ε, foetal γ, or adult δ/β in human; embryonic βh1/εY or adult βmaj/βmin in mice) to form a haemoglobin tetramer [5,6]. In humans, these tetramers include the embryonic haemoglobins Hb Gower 1 ($\zeta 2\mathcal{E}2$), Hb Gower 2 ($\alpha 2\mathcal{E}2$) and Hb Portland 1 ($\zeta 2\gamma 2$), the foetal haemoglobin HbF ($\alpha 2\gamma 2$), and the adult haemoglobins HbA ($\alpha 2\beta 2$) and HbA2 ($\alpha 2\delta 2$), representing 97% and <3% of adult haemoglobin, respectively [7]. In a study performed by Azevedo Portilho and colleagues [8], primitive haematopoietic cells were detected at 10.5 and 11.5 days in mouse placenta using histological and immunofluorescence techniques [8]. This investigation demonstrated that definitive erythrocytes are present in the maternal blood vessels while primitive erythrocytes are found in foetal channels. Foetal erythrocytes are characterised by an irregular shape, are larger than adult erythrocytes with intense cytoplasm due to the Hb content, and have a compact nucleus [8].

The function, structure and progression of primitive haemoglobins through to the adult haemoglobins have been extensively studied [9]. Mammalian Hb functions not only include transportation of gases but reportedly also scavenging of oxidizing agents [10,11] and protection of cells from oxidative and nitrosative stress [11,12]. Notably, Hb or subunits thereof have been revealed to be ectopically expressed in various non-erythroid cells such as neurons [13], lens and cornea [14], as well as in tumours such as non-Hodgkin’s lymphoma [15-17], glioblastoma [18], and breast cancer [19,20].

Haemoglobin is a molecule of fundamental importance in biology. However, robust detection of epsilon Hb in the placenta during development by immunohistochemistry (IHC) has not been well documented. The aim of this study was to develop and validate an immunohistochemical protocol for the specific detection of epsilon Hb using a human anti-Hb epsilon (HBE) antibody on primitive (nucleated) erythroid cells of the mouse placenta during the first trimester.

Materials and Methods

Mouse embryo with placental tissue

Eleven and a half day (d11.5) fixed outbred Swiss mouse embryos

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with associated placenta were obtained from the Animal Resource Centre (Murdoch, WA, Australia) in 10% buffered formalin solution.

DAB staining

Staining with DAB (3,3'-Diaminobenzidine) was performed using a protocol modified from Oosterwijk and colleagues [21]. Formalin-fixed paraffin-embedded (FFPE) mouse embryo tissue sections (4 µm) were deparaffinised in xylene and rehydrated using graded alcohol solutions to water. Slides were placed in 1 drop of DAB in chromogen solution (1-5% Biphenyl-3,3,4,4-tetrayltetraammonium tetrachloride) mixed with 1 mL buffer (Imidazole-HCl buffer, pH 7.5, containing hydrogen peroxide and an antimicrobial agent) per section for 3 hours in a dark environment. Slides were washed twice with tap water, counterstained using Harris hematoxylin for 1 min, then rinsed with tap water 3 times and dipped (15 seconds) in Scott's solution. The slides were washed twice, dehydrated, cleared, and mounted with a glass cover-slip.

Immunohistochemistry

The FFPE mouse embryo tissue sections (4 µm) on HURST® green slides (Hurst Scientific, Australia) were deparaffinised in xylene and rehydrated using graded alcohol solutions to water. Heat-induced antigen retrieval was performed by boiling in Tris/EDTA buffer (pH 9.0) followed by heating in a microwave oven (220-2200 W) sequentially on reheat-70°C (4 min X1), medium (4 min X1), and low (4 min X3), before cooling under running tap water. Sections were subjected to endogenous peroxidase quenching (3% H_2O_2 tap water for 30 min) and washed twice with tap water followed by 0.05 M Tris-buffered saline (TBS; pH 7.8). Sections were then blocked for unspecific proteins (protein block serum-free; Dako, North America, Carpinteria, USA) for 10 min at room temperature and treated with 0.2 M NaCl solution for 10 min to reduce ionic interactions and non-specific background staining due to endogenous peroxides that can arise from using mouse antibodies on mouse tissue. Immunohistochemistry was performed with an affinity-purified rabbit polyclonal antibody against amino acid residues 55-83 of the human embryonic haemoglobin epsilon (HBE) (ab156041, Abcam, Cambridge UK) at 1:1000 in antibody diluent (Dako) for 1 hour in a dark environment. Sections were then washed and incubated with a dual secondary goat anti-mouse/rabbit antibody (EnVision+ Dual Link System-HRP; Dako) for 30 min at room temperature. Reactions were developed with a DAB-chromogen solution containing 3,3` diaminobenzidine in 1-5% Biphenyl-3,3,4,4-tetrayltetraammonium tetrachloride, with substrate buffer (Imidazole-HCL buffer, pH 7.5 containing hydrogen peroxide (DAB; Dako) before being counterstained with Harris haematoxylin, dehydrated, cleared, and mounted with a glass cover-slip. A negative control was also included using the same tissue but with TBS.

Results

DAB histochemical staining was performed to detect all Hb on d11.5 developing mouse placenta sections. This identified both non-nucleated, circulating erythrocytes and nucleated, blood island cells as definitive and primitive erythroid cells, respectively as shown in Figure 1A and 1B. Using the IHC protocol, we specifically detected Hb epsilon in the mouse placenta sections as shown in Figure 1C and 1D.

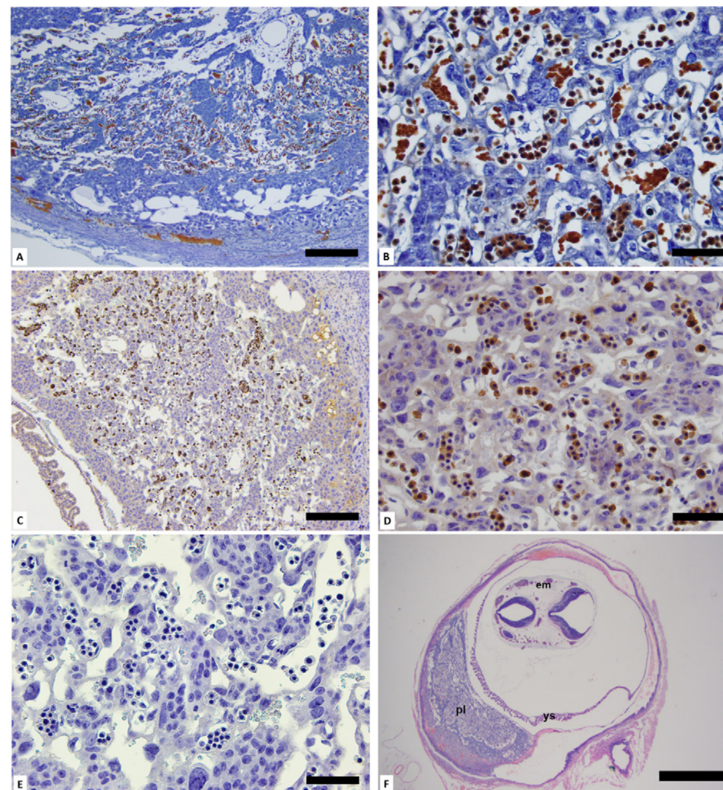

Figure 1: Specific detection of globin epsilon in 11.5d mouse placenta blood islands. (A and B) DAB histochemical staining for all Hb revealing both circulating definitive erythrocytes and primitive erythroid cells in placenta blood islands, magnification 10x, and 40x, respectively. (C and D) IHC for globin epsilon showing cytoplasmic immunopositivity specifically within primitive erythroid cells, magnification 10x, and 40x, respectively (brown=positive staining). (E) IHC performed without primary antibody as a negative control, magnification 40x. (F) H&E staining of an 11.5d mouse embryo reveals the chorionic cavity, placenta (pl), yolk sac (ys) and embryo (em), magnification 1.25x.

Haemoglobin epsilon was found to be present as dark brown staining exclusively in the cytoplasm of primitive (nucleated) erythroid cells of the placenta blood islands. By contrast, non-erythroid cells comprising the placental endothelium and mesothelium, as well as definitive erythrocytes, were consistently immunonegative for Hb epsilon. Minimal background staining was observed within the placenta endothelial and mesothelial cells as shown in Figure 1C and 1D, neither was staining observed in the absence of primary HBE antibody as shown in Figure 1E. Low magnification view stained with hematoxylin and eosin (H&E) shows the overall architecture of the embryo (em) and chorionic cavity, including the placenta (pl) and the yolk sac (ys) as shown in Figure 1F.

Discussion and Conclusion

We developed an IHC protocol using a commercially available polyclonal antibody directed against residues 55-83 of the human HBE. This 29-amino acid region of human HBE showed a predicted 72.4% and 79.3% with the equivalent regions of mouse embryonic Hb proteins βh1 and εY, respectively, which indicated that this antibody was likely to show cross-reactivity between these two species. During development, HBE is expressed specifically during primitive erythropoiesis in blood islands of the visceral placenta before being replaced by definitive erythropoiesis in foetal liver and bone marrow. Embryonic Hb belongs to a family of oxygen-carrying molecules with important physiological roles in erythroid cells. More recently, various Hb molecules have gained attention due to emerging evidence for their role in non-erythroid cells [13,19,22-26], and for their aberrant expression in various types of cancer [18,20,27-32]. The biological significance of this expression in many cases remains unclear, although Hb molecules can have various functions, not only for oxygen and carbon dioxide transport [23,33] but also as a nitric oxide scavenger [34,35] as well as in anti-oxidative stress, which been reported to enhance cell growth and reduce peroxidation induced by intracellular reactive oxygen species [11-13].

This study describes a reliable and specific IHC method for detecting Hb subunit epsilon in tissue sections based on a commercially available cross-reactive anti-human HBE-specific polyclonal antibody. Using this reagent, staining of HBE was successfully demonstrated in the cytoplasm of nucleated erythroid cells of placenta blood islands within d11.5 murine embryos. To our knowledge, such robust IHC detection of Hb epsilon using anti-HBE antibody in the developing placenta has not been previously reported. Our results are also consistent with the previously reported timeline and location of erythropoiesis in the mouse [8,36-38], with co-existence of primitive and definitive erythropoiesis in the mid-gestation embryo. In mice, primitive erythroid cells commence in yolk sac from 7.5 d and are not extinguished until 16.5 d, while definitive erythrocytes first appear in blood at 11.5 d [38].

This IHC technique for the demonstration of HBE in tissue sections can be utilised as an effective tool for research in a variety of settings, including haemopoiesis during development, as well as in non-erythroid tissues. Moreover, this IHC assay can be used in future studies to investigate the ectopic expression of Hb epsilon in different diseases states, including various neoplastic disorders.

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