Expression of haemopexin receptors by cultured human cytotrophoblast

Hans P. VAN DIJK,*‡ Martin J. KROOS,* Jaap S. STARREVELD,* Henk G. VAN EIJK,* Shao-Ping TANG,† De-Xiu SONG† and Ursula MULLER-EBERHARD†

*Department of Chemical Pathology, Erasmus Universiteit Rotterdam, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands and †Department of Pediatrics, Biochemistry and Pharmacology, Cornell University Medical College, New York, NY 10021, U.S.A.

The expression of cell-surface haemopexin (Hx) receptors on human cytotrophoblasts was assessed by using four different Hx species purified from plasma: human Hx isolated by wheatgerm-affinity chromatography, human Hx isolated by haem–agarose-affinity chromatography and rabbit and rat Hx, also isolated by haem–agarose-affinity chromatography. About 3500–7000 high-affinity (K_d 0.34–0.85 nM) receptors per cell were measured by Scatchard-type analysis at 4 °C using human (species obtained by both methods) or rabbit ^{125}I-labelled haem–Hx. Measured simultaneously, transferrin receptor number and affinity were 40000/cell and 0.83 nM respectively. In contrast with transferrin receptors, the number of Hx receptors did not increase during 24 h in cytotrophoblast culture. Rat Hx showed no specific binding to human Hx receptors in cytotrophoblast cultures.

INTRODUCTION

Many cell types {hepatocytes [1], HepG2 hepatoblastoma cells [2], K562 erythroleukaemic cells [3], HL-60 cells [4] and trophoblast (membrane) [5]} have been reported to express haemopexin (Hx) receptors. However, the number of receptors is very variable, and some reports [6,7] have even indicated that there are none. It has been suggested that the variation in the purity of the Hx prepared by different methods is the cause of the variations in the results obtained. Another observation that human and rabbit Hx bind more readily than rat Hx *in vivo* to rat (homologous) liver cells [8] requires explanation. To answer these questions, we have used Hx purified from the plasma of humans (prepared by two different methods), rabbits and rats to determine the number of high-affinity Hx receptors on human cytotrophoblast cells. The number of transferrin receptors (TfRs), which is well established [9–12], served as a positive control. Such a comparison is of particular interest because of the finding that haem–Hx down-regulates the expression of TfR [13].

MATERIALS AND METHODS

Chemicals

Ca^{2+} and Mg^{2+}-free solutions of Earle's balanced salts solution, fetal calf serum, Dulbecco's modified Eagle's medium with 20 mM Hepes, Medium 199, penicillin, streptomycin and amphotericin were obtained from Flow Labs (ICN Biomedicals, Zoetermeer, The Netherlands). Gentamicin was from Schering, trypsin (1:250) from Sigma and DNAase grade II from Boehringer-Mannheim. Haemin was purchased from Kodak (Transmedico, Weesp, The Netherlands), and $Na^{125}I$ was from Amersham.

Hx preparations

Human Hx was purified by haem–agarose-affinity chromatography [14] or wheatgerm-affinity chromatography [15] (details given in [14]); the latter procedure purifies Hx on account of its carbohydrate content. The Hx species obtained by the two methods are designated Hx(HA) and Hx(WG) respectively. Rat Hx was purified by haem-affinity chromatography, and rabbit Hx, purified by ion-exchange and size-exclusion chromatography, was a gift from Dr. Ruba S. Deeb and Dr. David H. Peyton [16]. All preparations were more than 95% pure, as judged by SDS/PAGE (applying 1–10 μg per lane) and testing by Western-blot analysis with anti-albumin and anti-transferrin antibodies, as these proteins are the most common contaminants encountered. Haem-binding ability was investigated by haem titration using the Soret peak of the haem–Hx complex, ascertaining the 1:1 binding ratio, and screening the quality of the complex by scanning spectrometry between 400 and 600 nm.

Formation of the haem–Hx complex

Haem solution was prepared by dissolving 0.1 mg of haemin in 100 μl of 0.1 M NaOH. To this burgundy red solution was added 100 μl of 0.1 M Pipes (sodium salt) buffer; this served as a stock solution and was stable for 1 h at 4 °C. Haem concentration was measured at 400 nm in 40% dimethyl sulphoxide as blank; absorbance corrected for the blank varied between 0.490 and 0.534. Protein concentration was measured at 280 nm using an absorption coefficient of 19.7 ($A^{1\%}_{1cm}$) [17]. Apo-Hx stock solution was diluted in PBS and loaded with haem, using a haem/protein ratio of 0.9:1. After a 1 h incubation at 37 °C, the mixture was passed over a Whatman DEAE-cellulose DE52 column, equilibrated and eluted with 15 mM sodium phosphate buffer, pH 7.4. The degree of saturation was measured by the A_{400}/A_{280} ratio. Haem saturation was always between 80 and 90%. The entire procedure was performed under dimmed light, the tubes being wrapped with aluminium foil.

Iodination of Hx

About 150–200 μg of haem–Hx was allowed to react for 10 min at room temperature with 0.5 mCi of $Na^{125}I$ in a glass vial coated with 100 μg of Iodogen (Pierce Chemicals Co., Rockford, IL, USA). Free iodine was removed from the haem–^{125}I-labelled Hx

Abbreviations used: Tf-2Fe, diferric transferrin; TfR, transferrin receptor; Hx, haemopexin; NTA, nitrilotriacetate.

‡ To whom correspondence should be addressed.

by passing the mixture over a disposable PD-10 Sephadex column, equilibrated and eluted with PBS, followed by extensive dialysis against PBS. The labelling took place under dimmed light; dialysis was performed in the dark at 4 °C.

Labelling of human diferric transferrin (Tf-2Fe)

Human Tf-2Fe was obtained by full saturation of human apotransferrin in 0.1 M Tris/HCl (pH 8.2)/$NaHCO_3$ in 10-fold molar excess of iron, using iron–nitrilotriacetate (Fe–NTA) in a molar ratio of 1 Fe:2 NTA. Excess Fe–NTA was removed on a PD-10 Sephadex column, followed by extensive dialysis against PBS. Human Tf-2Fe (0.5 mg) was labelled with $Na^{125}I$ by the Iodogen method (see the preceding section). Excess ^{125}I was removed as described above.

Cytotrophoblast isolation

Human placentas from healthy women were obtained from the Department of Obstetrics, University Hospital Dijkzigt, Rotterdam, The Netherlands within 30 min of spontaneous delivery. Trophoblast cells were isolated by the slightly modified procedure of Kliman et al. [18]: approx. 30 g of villous tissue was pre-incubated for 15 min at 37 °C with 50 ml of the enzyme solution used subsequently for the successive steps of the Kliman procedure. The supernatant was discarded. The remaining tissue was processed as described by Kliman et al. [18]. The final cell population consisted of at least 95% cytotrophoblasts [9].

Cell-culture conditions

Isolated cells were counted (Bürker counting chamber), diluted to 6×10^5 cells/ml in culture medium, and plated in 35 mm Falcon culture dishes (Greiner and Söhne, Freiburg, Germany). The final density was 1.5×10^6 cells/dish. Culture medium consisted of 80% (v/v) Medium 199, 20% (v/v) fetal calf-serum, 4 mM L-glutamine, 0.3 mg/ml gentamicin, 50 units/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml amphotericin. The osmolarity was 280–300 mosmol/kg and the pH was 7.4. Before use the medium was sterilized by filtration on a 0.22 μm Millipore-GS filter. Cultures were incubated at 37 °C in humidified 5% CO_2/95% air. After 24 h of recovery, the non-adherent cells were removed by washing the dishes twice with culture medium. The culture medium was renewed every 24 h. After a further 24 or 48 h, the cells were used in the binding experiments.

Binding studies and Scatchard-plot analysis

All binding studies were performed under dimmed light at 4 °C. The dishes, containing single cells, cell aggregates and numerous syncytia, were washed twice with ice-cold assay medium consisting of Hanks medium supplemented with 25 mM Hepes and 0.2% ovalbumin. Binding studies were performed in 1 ml of assay medium. The ^{125}I-labelled haem–Hx concentrations used ranged from 0.015 to 4.2 μg/ml. Non-specific binding was estimated in the presence of a 100-fold excess of unlabelled homologous haem–Hx. After 1 h of incubation at 4 °C (equilibrium binding was reached between 30 and 45 min), the cells were washed three times with cold PBS and then lysed in 1 ml of 0.1 M NaOH. The cell material was collected, and cell-associated radioactivity determined.

One placenta yields 40×10^6–60×10^6 primary mononuclear cells, which is enough to obtain 30–40 data points. As all data points are mean values of two or three determinations, non-specific binding could not be assessed systematically. We therefore estimated non-specific binding by a computerized iteration method. This method is based on a stepwise increase in a linear non-specific-binding component. Iteration stops when the Scatchard plot of the specific binding data gives optimal correlation. Correlation coefficients between −0.93 and −0.96 were obtained. Parallel binding studies with human Tf-2Fe were performed in the same manner. Non-specific binding was simultaneously measured with a 100-fold excess of unlabelled human Tf-2Fe.

RESULTS

The results of a representative binding experiment performed with human Hx(HA) are shown in Figure 1. Non-specific binding was determined by iteration, as described in the Materials and methods section. The experimentally obtained non-specific binding data (not shown) were in good agreement with the theoretically obtained data. In the lower range of the binding isotherm, non-specific binding did not exceed the 5% level. In the upper range, non-specific binding approached the 80% level. Specific binding is limited; only 3500–7000 binding sites per cell were measured, but the affinity constant was high (K_d = 0.34–0.83 nM).

Figure 2(a) shows the Scatchard plot of the specific binding data shown in Figure 1 as well as a plot of the data obtained from an experiment performed with human Hx(WG). In the latter experiment the cell yield allowed us to perform simultaneously a binding experiment with human Tf-2Fe. Scatchard transforms of the experiment with human Tf-2Fe as well as that of human Hx(WG) are given in Figure 2(b). From these data we derive the number of human Tf-2Fe-binding sites to be 3.5×10^4 per cell and the K_d to be 0.72 nM. For human Hx(WG) the number of binding sites was 7000 per cell, and the K_d 0.55 nM; these values were similar to those obtained for human Hx(HA) (Figure 1).

In Table 1 the results obtained for all species of Hx tested are listed. Whereas the data for rabbit Hx were very similar to those for the two human Hx preparations, those for rat Hx showed no specific binding. Two additional experiments with a new batch of rat haem–Hx gave the same results which excludes

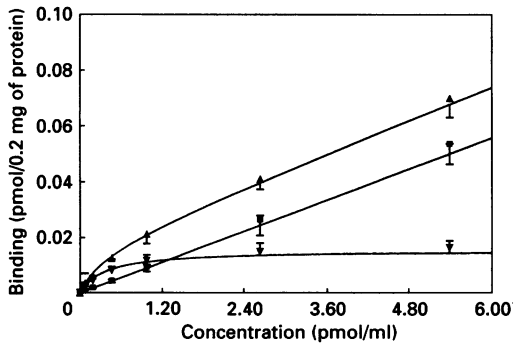

Figure 1 Binding isotherms of human haem–Hx(HA) to syncytiotrophoblasts

Total binding, non-specific binding and specific binding of human haem–Hx(HA) to 70 h-cultured cytotrophoblasts are shown. The curves are based on mean values of three independent experiments performed in duplicate. Error bars represent the S.D., calculated from three averaged data points. The symbols represent data obtained in one experiment; each data point is the mean of two estimates. The Scatchard plot of this particular experiment is given in Figure 2. ▲, Total binding; ■, non-specific binding; ▼, specific binding.

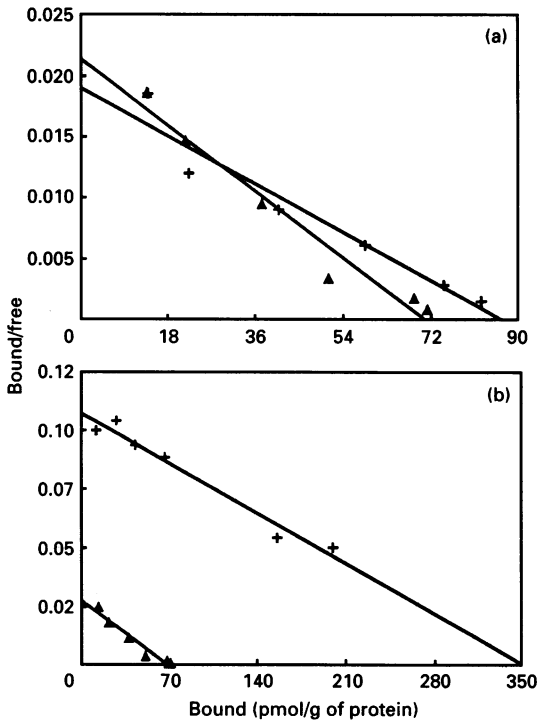

Figure 2 Scatchard analysis of binding of human haem–Hx(HA and WG) and human Tf-2Fe to syncytiotrophoblasts

(**a**) Scatchard plots of two experiments (cytotrophoblast cells isolated from different placentas, cultured for 70 h) are shown. +, experiment with human haem–Hx(HA); ▲, experiment with human haem–Hx(WG); $K_d^{HA} = 0.45$ nM; $K_d^{WG} = 0.55$ nM. The corresponding numbers of Hx-binding sites are 6900 and 7000 per cell. The differences are not significant (see Table 1). (**b**) Binding of human Tf-2Fe and human haem–Hx(WG) were assessed simultaneously in the same cytotrophoblast population, cultured for 70 h. The dissociation constants are nearly equal, but the number of binding sites per cell is different [3.5×10^4 for human Tf-2Fe and 7000 for human Hx(WG)]. +, human Tf-2Fe; ▲; human haem–Hx (WG).

Table 1 Effect of culture time and Hx species on affinity constant and receptor number

All measurements were made 24 h after the first medium change, except those indicated (*) which were made 48 h after the first medium change. Neither the dissociation constant K_d nor the receptor number per cytotrophoblast differed significantly when compared using the Student–Newman–Keuls test, which allows multi-comparisons. —, No specific binding detectable.

Hx species	No. of independent experiments	K_d (nM)	Receptor no./ cytotrophoblast
Human WG	3	0.47 ± 0.13	4400 ± 1160
Human HA	4	0.58 ± 0.16	6500 ± 600
Rabbit	3	0.61 ± 0.16	4800 ± 950
Rabbit*	4	0.78 ± 0.29	3900 ± 1150
Rat	5†	–	–

† Included two control experiments.

the possibility that the lack of binding was the result of damaged Hx. The number of binding sites for the human and rabbit Hx species was not significantly different when compared by the Student–Newman–Keuls test.

DISCUSSION

Cytotrophoblasts isolated from term placentas and cultured in Medium 199 differentiate biochemically as well as morphologically into syncytiotrophoblast-like structures. Part of this biochemical differentiation is the expression of TfRs [9]. The degree of TfR expression depends on culture time and culture conditions. Iron-poor culture medium stimulates TfR expression. After 48 h of culture in iron-poor Medium 199 the number of surface TfRs amounted to 30000–45000 per cell (0.3–0.5 pmol/mg of cell protein) [10,11].

The results of our Hx-binding studies suggest the presence of a very limited number of Hx receptors at the syncytiotrophoblast cell surface (3500–7000 per cell). The measured affinities range from 0.34 to 0.83 nM and are comparable with values published by Taketani et al. [13] for HeLa cells, but are 30-fold higher than those reported by Taketani et al. [5] for isolated placental brush-border membranes.

One must bear in mind that the isolated cell population consists of about 95% cytotrophoblasts and 5% contaminating cells (mainly monocytes). An explanation for the small receptor number might be that the Hx receptors detected are associated not with syncytiotrophoblasts but rather with the approx. 5% non-trophoblast cells in our culture dishes. On the other hand, Hx receptors can be expected on all epithelial cells that predominantly remove the haem–Hx complex [19].

In contrast with TfR expression, no effect of culture time on Hx-receptor expression could be detected. Trophoblast cells cultured for only 24 h gave essentially the same results as those cultured for 48 h (Table 1).

Figure 2(b) demonstrates that the low Hx-receptor expression is not due to the method of cell isolation and culture conditions. TfR expression at 48 h and TfR affinity are in the expected range (Tf-2Fe binding 0.26 pmol/mg of protein; K_d 0.67 nM).

We conclude that high-affinity Hx receptors can be detected in trophoblast cell cultures. It was not determined whether they are localized on the syncytiotrophoblast surface. The methods used for the isolation of human Hx did not influence Hx-binding characteristics. Rabbit Hx bound almost as well as the human species, but rat Hx failed to interact with cultured cytotrophoblasts. The most likely explanation for this failure is a particular species-specific structure present in the rat Hx molecule.

The work was supported by NIH grant DK30203 and the Robert Leet and Clara Guthrie Patterson Trust.

REFERENCES

1 Smith, A. and Morgan, W. T. (1981) J. Biol. Chem. **256**, 10902–10909
2 Smith, A. and Hunt, R. C. (1990) Eur. J. Cell. Biol. **53**, 234–245
3 Taketani, S., Kohno, H. and Tokunaga, R. (1986) Biochem. Int. **13**, 307–312
4 Taketani, S., Kohno, H. and Tokunaga, R. (1987) J. Biol. Chem. **262**, 4639–4643
5 Taketani, S., Kohno, H., Naitoh, Y. and Tokunaga, R. (1987) J. Biol. Chem. **262**, 8668–8671
6 Sinclair, P. R., Bement, W. J., Gorman, N., Liem, H. H., Wolkoff, A. W. and Muller-Eberhard, U. (1988) Biochem. J. **256**, 159–165
7 Sinclair, P. R., Bement, W. J., Healey, J. F. et al. (1995) Hepatology, in the press
8 Smith, A. and Morgan, T. (1979) Biochem. J. **182**, 47–54
9 Bierings, M. B., Adriaansen, H. J. and van Dijk, J. P. (1988) Placenta **9**, 387–396
10 Bierings, M. B., Beart, M. R. M., van Eijk, H. G. and van Dijk, J. P. (1991) Mol. Cell. Biochem. **100**, 31–38
11 Starreveld, J. S., van Dijk, J. P., Kroos, M. J. and van Eijk, H. G. (1993) Clin. Chim. Acta **220**, 47–60
12 Douglas, G. C. and King, B. F. (1990) Placenta **11**, 41–57
13 Taketani, S., Kohno, H., Sawamura, T. and Tokunaga, R. (1990) J. Biol. Chem. **265**, 13981–13985
14 Muller-Eberhard, U. (1988) Methods Enzymol. **163**, 536–564
15 Vredblad, P. and Hjorth, R. (1977) Biochem. J. **167**, 759–764

16 Deeb, R. S., Muller-Eberhard, U. and Peyton, D. H. (1994) Biochim. Biophys. Acta **1200**, 161–166
17 Seery, V. L., Hathaway, G. and Muller-Eberhard, U. (1972) Arch. Biochem. Biophys. **150**, 269–272
18 Kliman, H. J., Nestler, J. E., Sermasi, E., Sanger, J. M. and Straus III, J. F. (1986) Endocrinology **118**, 1567–1582
19 Potter, D., Chroneos, Z. C., Baynes, J. W., Sinclair, P. R., Gorman, N., Liem, H. H., Muller-Eberhard, U. and Thorpe, S. R. (1993) Arch. Biochem. Biophys. **900**, 98–104

Received 1 December 1994/accepted 14 December 1994