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Methaemoglobin enhances the proliferation of transformed human epithelial cells: a possible outcome of neovascularisation and haemorrhage in tumours?

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Abstract

The effect of human methaemoglobin (metHb), possibly derived from extravasated red blood cells in tumours showing neovascularisation and haemorrhage, on the growth of transformed human epithelial cells was investigated. MetHb stimulated the growth of immortalised epithelial cells or transformed cells at precrisis stage (cells have bypassed M1, but not M2, the two mortality checkpoints). The stimulatory effect was due to the release of haemin from metHb that was isolated by a Sephadex column and identified by its characteristic light absorption spectrum. Although all the degradation products of haemin are currently known to be physiologically significant, only ferric iron derived from metHb or haemin could stimulate cell growth. High concentrations of metHb or haemin inhibited cell growth possibly due to the generation of high concentrations of bilirubin. However, bilirubin formed in the cells of human body is known to be transported to the liver for further processing and excretion. Haemoglobin oxidised to where tumours show neovascularisation and haemorrhage likely contributes significantly to the increased proliferation of cancerous cells.

Keywords: Neovascularisation; Haemorrhage; Methaemoglobin; Iron; Tumour growth

1. Introduction

Abnormal vasculature showing leaky walls and haemorrhage is a common feature of malignant tumours [1]. Clinically, magnetic resonance imaging can often identify the presence of methaemoglobin (metHb) in various solid tumours [2,3]. Previous studies demonstrated that ferrohaemoglobin in solution or contained in red blood cells can be oxidised to metHb by activated inflammatory cells [4,5], suggesting that immunological surveillance may play a role in generating metHb in tumours *in vivo*. In the metHb molecule, ferrihaem is not associated by a covalent bond and may therefore be released under physiological conditions. In fact, ferrihaem has been shown to undergo exchange among human haemoglobins and even between human haemoglobin and primate albumin [6]. Haemin or haem in the microsomal fraction of cells is then degraded by a complex enzyme system, haem oxygenase (HO), to form biliverdin, carbon monoxide and ferric iron.

Biliverdin and its reduction product, bilirubin, are two potent antioxidants [7]. Carbon monoxide is currently believed to be, like nitric oxide, a signal molecule related to cyclic 3′,5′-guanosine monophosphate (cGMP) and prostaglandin E2 (PGE_2) productions in different biological systems [8–11]. Iron is an essential element for the growth and viability of all cells [12]. MetHb sensitises human umbilical vein endothelial cells to oxidant-mediated cytotoxicity [13]; the same result was also observed in human breast cancer cells exposed briefly to haemin [14]. The destructive effect of metHb or haemin is probably due to the fact that haem degradation is a potential generator of hydroxyl radicals and other reactive oxygen species that may lead to tissue damage related to the pathology of ischaemic reperfusion of the kidney, heart and brain [15].

The purpose of the present study was to determine whether metHb derived from extravasated red blood

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cells could stimulate or inhibit tumour cell proliferation. To examine this, it was necessary to grow cells in the absence of serum, which contains, batch to batch, various amounts of haemoglobin. In addition, the majority of human cancers originate from epithelial cells. In this study, therefore, cultures of normal human nasal and nasopharyngeal epithelial cells transformed by transfection with human papillomavirus (HPV) type 16 DNA in a chemically-defined medium previously established in my laboratory [16], were used at early or late passages to determine their responses to various doses of human metHb or haemin. The effect of human metHb in a serum-free medium on the growth of a subclone of cancer cell line HeLa, HeLa-W, was also assessed. Experiments were then carried out to explore the mechanism through which human metHb acts. In this report, I discuss the significance of the results in the context of tumour cell growth, vasculature and haemorrhage.

2. Materials and methods

2.1. Cell cultures

The cell line NW-1 was established from epithelial cells of human nasal polyps transfected with the total HPV type 16 DNA [16]. The cell line was maintained in basal medium (MCDB-151 containing 0.1 mM calcium chloride) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 1.6 nM epidermal growth factor (EGF), 0.87 μM insulin and 30 μg/ml bovine pituitary extract in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. NW-1 cells used here have passed the stages of crises (M1 and M2) and should be regarded as an immortal cell line for they have reached greater than 200 population doublings [17]. NPC-N cells were epithelial cells of human nasopharyngeal origin also transfected with the total HPV type 16 DNA that resulted in an extended life span [16]. NPC-N cells used here have not passed M2 and were also maintained in the aforementioned undefined medium containing 30 μg/ml bovine pituitary extract. HeLa-W, a subclone of the HeLa cell line, was seeded in 1% Vitrogen 100-coated dishes (Celtrix Laboratories, Palo Alto, CA, USA) and cultured in Ham's nutrient mixture F12.

NPC-N cells at passages 33–42 and NW-1 cells at passages $\geqslant 224$ were seeded in triplicate in the basal medium containing 1.6 nM EGF and 0.87 μM insulin (and where stated in the figure legends) the seeding medium additionally contained 0.15 μM haemin at a density of 20 000 cells/3.5-cm culture dish overnight before undergoing different treatments. HeLa-W cells were also plated in triplicate, but in F12 at a seeding density of 10 000 cells/3.5-cm culture dish overnight for later treatment. Culture media were replenished every 2–3 days, depending on the cell density. The cultures of HeLa-W cells were terminated at 7 days, whereas NPC-N and NW-1 cells were terminated 2 weeks after plating by trypsinisation. Growth was estimated by counting the cells using a Coulter counter (Coulter Electronics Ltd., Beds, UK) or haemocytometer after the cells were stained with trypan blue. Cell yields from the cultures maintained in seeding media served as growth controls for computing the relative cell growth: NW-1 cells in seeding medium with or without 0.15 μM haemin yielded $(14.3\pm0.6)\times10^4$ and $(2.8\pm0.3)\times10^4$ cells, respectively, NPC-N cells in seeding medium generated $(0.4\pm0.1)\times10^4$ cells; HeLa-W in seeding medium produced $(4.2\pm0.1)\times10^4$ cells. Data were compared using the Student *t*-test. Results were considered statistically significant when $P<0.05$.

2.2. MetHb preparation and purification

100 mg of human haemoglobin (Sigma Chemical Co.) was dissolved in 10 ml of 67 mM sodium phosphate buffer, pH 6.6 and centrifuged at 15 000*g* for 15 min at 4 °C. The resulting supernatant was mixed with a 1.25-fold excess of potassium ferricyanide, and was allowed to stand at room temperature for 10 min for conversion of residual ferrohaemoglobin to metHb. Ferrocyanide was removed by loading the reaction mixture into a Sephadex G-25 (Amersham Pharmacia Biotech, Taipei, Taiwan) column eluted with 50 mM sodium chloride. The metHb prepared was analysed by two-dimensional polyacrylamide gel electrophoresis and the purity of it was estimated to be 99.5% using the conventional non-diamine silver staining method to detect proteins in the gel. For cell cultures, metHb in 50 mM sodium chloride was sterilised by filtration using a Millex-GV filter unit (Millipore, Co., Bedford, MA, USA) and mixed thoroughly with an equal volume of 2× concentrated basal medium. The concentration of metHb was determined using Bradford's method.

2.3. Globin preparation and purification

Globin was prepared from human haemoglobin by acid-acetone method as described in Ref. [18], then purified from Sephadex G-25 column and filtered for cell cultures as described above.

2.4. Identification and quantification of haemin release

15 mg metHb freshly isolated from a Sephadex G-25 column in 13-ml basal medium either with or without an equal mole number of Chicken IgY antibody against human metHb (see below for its preparation) was distributed to each of eight 10-cm dishes. The dishes were then placed in a CO_2 incubator set to the conditions for

culturing NW-1 cells. At different incubation times, the contents of the dishes were loaded onto a 1×4 cm plastic column filled with medium-sized Sephadex G-25. The column was first extensively eluted with 50 mM NaCl until no absorption was detected at 280 nm, then eluted with dimethyl sulphoxide to elute out fractions separately containing protein and the gel-bound haemin. The haemin from the column was identified by its light absorption spectrum scanned from 260 to 700 nm and the absorption spectrum in that range obtained from authentic haemin dissolved in dimethyl sulphoxide served as a standard for comparison. The amount of haemin released from metHb was subsequently determined from its light absorption at 403 nm using different amounts of the authentic haemin dissolved in dimethyl sulphoxide for calibration, and later normalised.

2.5. Chicken IgY antibody against human metHb

Chicken IgY antibody that recognises human metHb were acquired from Leghorn hens, 24 weeks old, immunised with bovine haemoglobin. The isolation of crude antibody from egg yolks using dextran sulphate was carried out in accordance with the published method [19]. The antibody was further purified by an affinity column packed with bovine haemoglobin which was linked beforehand to 4% agarose bead using cyanogen bromide (Sigma Chemical Co.). For cell cultures, antibody purified from affinity columns was further run through one Sephadex G-25 column equilibrated with 50 mM sodium chloride to remove cytotoxic substances such as TRIS. The antibody dissolved in 50 mM sodium chloride was filtered through a Millex-GV filter unit and finally mixed thoroughly with an equal volume of 2× concentrated basal medium for cell cultures.

2.6. Western blot analysis

0.5 μg bovine or human haemoglobin separated by 20% sodium dodecyl sulphate polyacrylamide gel electrophoresis was transferred to a piece of Immobilon-P membrane (Millipore, Co, Bedford, MA, USA) by wet transfer. In a 20% gel, the α-subunit of bovine haemoglobin, α-subunit of human haemoglobin, β-subunit of bovine haemoglobin and β-subunit of human haemoglobin migrated closely to each other in the order just mentioned towards the anode. The transferred membrane was cut into six lanes. Each of them was incubated with an equal amount of the aforementioned chicken IgY antibody prepared for cell culture and different amounts of human metHb, followed by incubation with peroxidase-labelled rabbit anti-chicken IgY (Sigma Chemical Co.). Immunodetection was performed using an enhanced chemiluminescence detection kit, ECL (Amersham Pharmacia Biotech.).

3. Results

Purified human metHb, but not its globin moiety, tested at the concentrations between 0.01 and 5 μM augmented the growth of all three types of transformed human epithelial cells (Fig. 1). The cell numbers were increased maximally by 12-, 6.7- and 4.2-fold ($P<0.001$) when NPC-N, NW-1 and HeLa-W cells were cultured in the presence of 0.6, 0.3 and 1.2 μM of human metHb, respectively, in comparison with the cell yields from cells cultured in seeding media (Fig. 1).

MetHb prepared for the aforementioned studies may contain an unidentified potent growth factor. To rule this out, chicken IgY antibody against human metHb was purposely generated from hens using bovine haemoglobin as an antigen. Fig. 2a shows that the antibody generated recognised both α- and β-subunits of the antigen (lane 1) and only the α-, but not the β-subunit, of human haemoglobin (lane 2), possibly due to a larger difference in amino acid sequence between the two types of β-subunit. The recognition of α-subunit of human haemoglobin was specific as the signal was blocked by human metHb (Fig. 2a, lanes 2–6). The same batch of

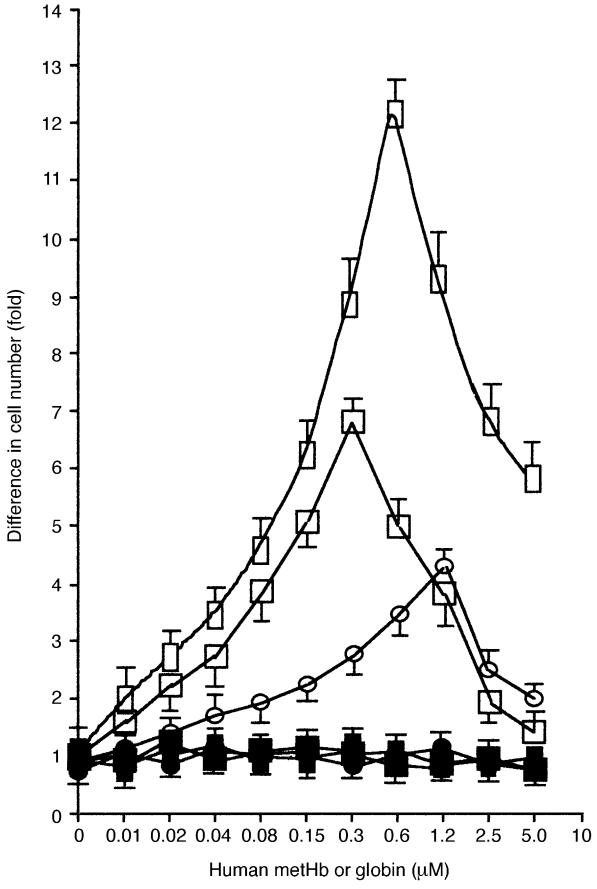

Fig. 1. Effect of human methaemoglobin (metHb) (empty symbols) and globin (filled symbols) on the growth of NPC-N (rectangles), NW-1 (squares) and HeLa-W (circles) cells. All values represent the mean of three independent experiments ± standard deviation (S.D.) (T).

chicken IgY antibody, diminished the metHb-induced growth of NW-1 cells (Fig. 2b). The reduced growth was not due to a cytotoxic effect inherent in the preparation of the antibody: the growth of NW-1 cells in medium supplemented with EGF and insulin versus that in medium containing EGF, insulin and the chicken IgY antibody was not statistically different (Fig. 2b, human metHb = 0 μM).

Haptoglobin is a molecule that binds haemoglobin [20]. Applying 0.06–0.44 μM haptoglobin to growing NW-1 cells was not cytotoxic for cells in a medium containing EGF and insulin and growth was not altered significantly (Fig. 3, column 1 versus columns 2 and 3). Notably, Fig. 3 indicates that the metHb-induced growth of NW-1 cells was reduced by using human pooled plasma haptoglobin (Sigma Chemical Co.) and was recoverable by using higher ratios of metHb over haptoglobin.

Fig. 4 shows that the optimal dose of human metHb alone to NW-1 and NPC-N cells was not growth-sti-

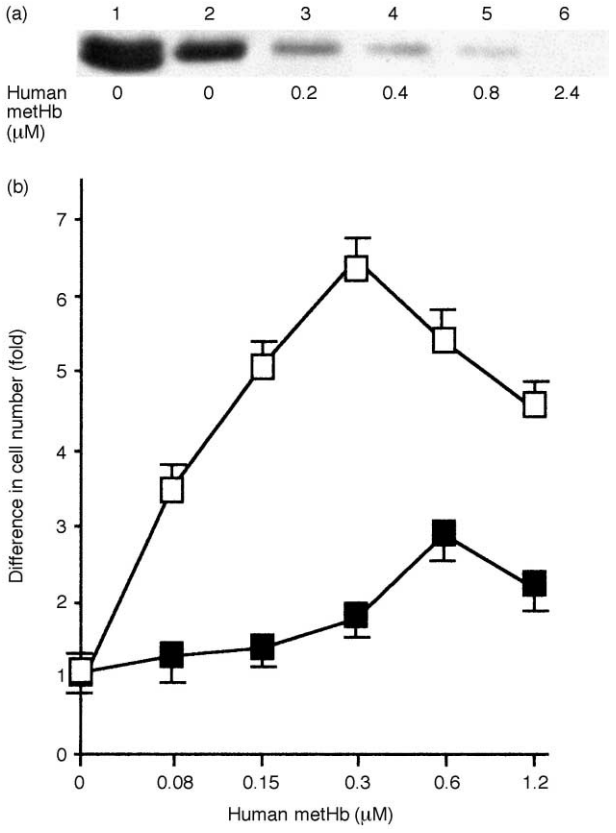

Fig. 2. (a) Immunoblotting analyses show that chicken IgY antibody against bovine haemoglobin (lane 1) recognises the α-subunit of human haemoglobin and the recognition is increasingly blocked by increased amounts of human metHb (lanes 2–6). (b) Chicken IgY antibody against bovine haemoglobin inhibits human methaemoglobin (metHb)-induced growth of NW-1 cells. White squares: absence of the antibody. Black squares: presence of the 0.3 μM antibody. All values represent the mean of two independent experiments ± standard deviation (S.D.).

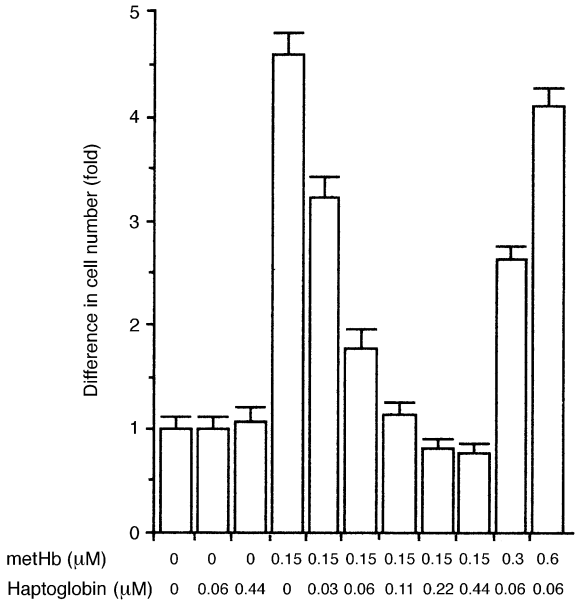

Fig. 3. Effect of haptoglobin on the human methaemoglobin (metHb)-induced growth of NW-1 cells. All values represent the mean of two independent experiments ± standard deviation (S.D.).

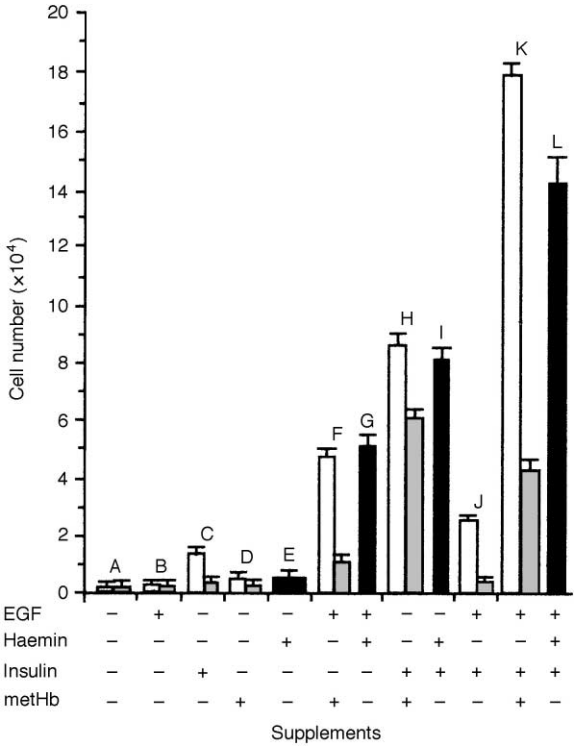

Fig. 4. Effect of the supplements on the growth of NW-1 (white and black columns) and NPC-N (dotted columns) cells. These cells were cultured in basal medium with (+) or without (−) a certain supplement as indicated under columns. Concentrations tested: epidermal growth factor (EGF), 1.6 nM; insulin, 0.87 μM; methaemoglobin (metHb), 0.3 μM; haemin, 0.15 μM. All values represent the mean of three independent experiments ± standard deviation (S.D.).

mulating (Fig. 4, D), but it did potentiate the mitogenic effects of EGF and/or insulin (Fig. 4, F, H and K).

Human metHb dissolved in basal medium and left in a CO_2 incubator released haemin that could be isolated by Sephadex G-25 column chromatography for further characterisation and quantification (Fig. 5 and inset). Authentic haemin and the free haemin isolated from Sephadex G-25 column showed identical light absorption spectrum at the wavelengths 260–700 nm and a distinct absorption maximum at 403–404 nm (Fig. 5). In a 3-day period of the incubation in the absence of the chicken antibody, haemin release increased with time and 3.1% of the theoretical haemin molecules in 15 mg human metHb became unbound. When the parallel experiment was conducted in the presence of equal mole number of the chicken antibody, haemin release did not rise and 0.9% of the theoretical haemin molecules in human metHb was released (Fig. 5, inset). Fig. 6 shows that haemin as an additional growth supplement in the culture medium behaved like metHb: there was no effect on cell proliferation at concentrations below 0.02 μM, whereas there was a concentration-dependent growth stimulation that reached a maximum of a 6.5-fold increase ($P<0.001$) at 0.15 μM, and an inhibition of cell growth at concentrations higher than 1.2 μM. Haemin alone, like metHb, was not growth-stimulating (Fig. 4, E), but also contributed significantly to the mitogenic effects of EGF and/or insulin (Fig. 4, G, I and L).

Fig. 6 also shows that protoporphyrin IX (ppIX), which is structurally similar to haemin, but possesses no iron, was not growth-stimulating, even though it was reported possibly to activate soluble guanylate cyclase (GCase) [21]. Biliverdin and PGE_2 at concentrations ranging from 0.01 to 1.2 μM added as an additional growth factor to medium already containing EGF and insulin also did not increase cell growth (Fig. 6). PGE_2 added at concentrations ranging from 0.1 nM to 0.01 μM also did not show any significant effect on cell growth (data not shown).

Haemin higher than 1.2 μM inhibited cell growth (Fig. 6). To determine whether the amount of ferric ion that was possibly generated from greater than 1.2 μM haemin concentrations was toxic to NW-1 cells, ferric nitrate or ferrous sulphate was added to the culture medium at concentrations up to 25.6 μM. Neither one of them inhibited cell growth, instead both could significantly increase the proliferation of NW-1 cells more than 10-fold ($P<0.001$) (Fig. 7).

Haemin-induced proliferation of NW-1 cells was abrogated by adding zinc-protoporphyrin IX (zn-ppIX), a specific haem oxygenase competitive inhibitor [22], or desferrioxamine (DFO), a membrane-permeable chelating agent with a very high affinity and specificity for ferric ion [23] (Fig. 8). However, the induced cell proliferation was not affected by the presence of 1H-(1,2,4)oxadiazolo[4,3-alpha]quinoxaline-1-one (ODQ), a selective soluble GCase inhibitor (Fig. 8) [24]. Fig. 8 also shows that bilirubin could inhibit cell growth,

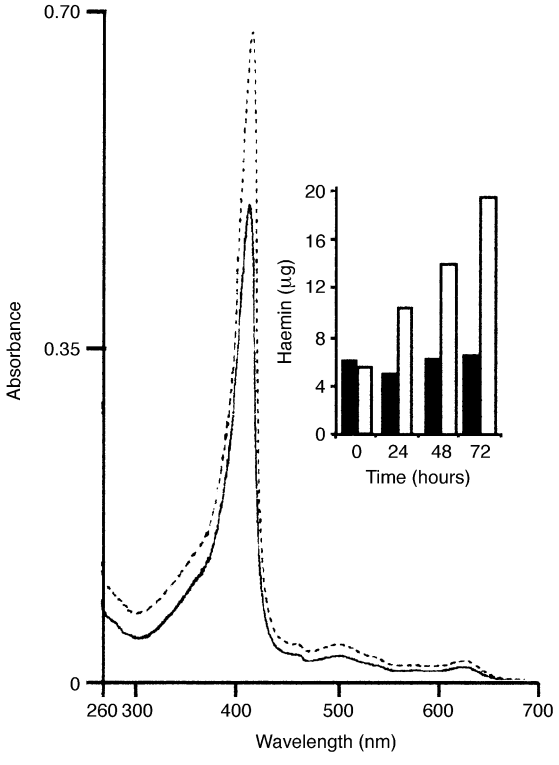

Fig. 5. Characterisation and quantification of haemin dissociated from human methaemoglobin (metHb). Broken and solid lines represent light absorption spectrum of authentic haemin and haemin dissociated and isolated from the Sephadex G-25 column, respectively. Inset shows the amount of haemin released in the presence (black columns) or absence (white columns) of the chicken antibody.

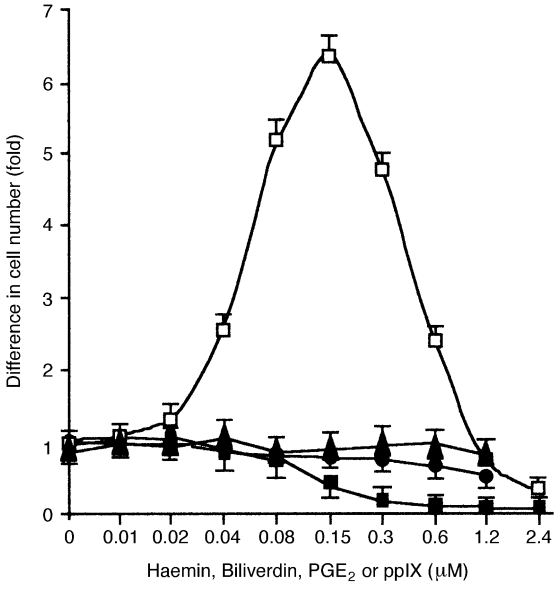

Fig. 6. Effect of haemin (white squares), biliverdin (triangles), prostaglandin E2 (PGE_2) (dots) or protoporphrin IX (ppIX) (black squares) on the growth of NW-1 cells. All values represent the mean of two independent experiments ± standard deviation (S.D.).

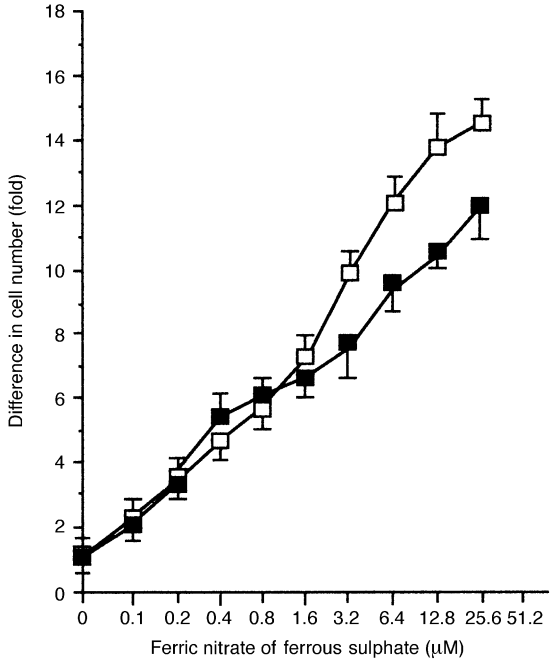

Fig. 7. Effect of iron supplement on the growth of NW-1 cells. Black squares: ferric nitrate nonahydrate. White squares: ferrous sulphate heptahydrate. All values represent the means calculated from the cell culture in triplicate ± standard deviation (S.D.).

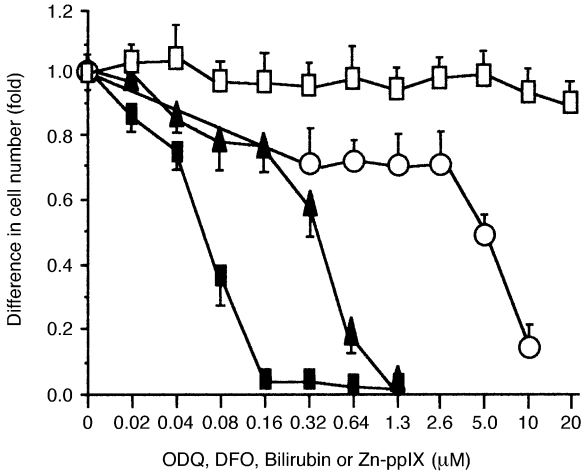

Fig. 8. Effect of ODQ (white rectangles), desferrioxamine (DFO) (circles), bilirubin (triangles) or zinc-protoporphyrin IX (zn-ppIX) (black rectangles) on the growth of NW-1 cells cultured in the basal medium supplemented with 1.6 nM epidermal growth factor (EGF), 0.87 μM insulin and 0.15 μM haemin. All values represent the means calculated from cell cultures in triplicate ± standard deviation (S.D.).

particularly at concentrations greater than 0.32 μM ($P < 0.001$).

One possible explanation for why haemin-induced cell proliferation was abolished by DFO or zn-ppIX is that these drugs probably make the iron inside the cell less available for cell replication. Table 1 shows that, even though haemin-induced cell growth was inhibited more than 90 and 50% by DFO and Zn-ppIX, respectively, it could be completely reversed by simultaneously adding ferrous or ferric ion to the culture medium.

Table 1
Ferrous sulphate heptahydrate and ferric nitrate nonahydrate restore the proliferation of NW-1 cells induced by haemin and abolished by DFO or Zn-ppIX. NW-1 cells were platted and cultured in the seeding medium or in the seeding medium supplemented additionally with the compounds indicated

Supplement	Relative cell yield[a]
None	1.00±0.14
80 nM haemin	4.64±0.21
80 nM haemin + 10 μM DFO	0.07±0.01
80 nM haemin + 10 μM DFO + 9.6 μM $FeSO_4 \cdot 7H_2O$	4.57±0.18
80 nM haemin + 10 μM DFO + 3.2 μM $Fe(NO_3)_3 \cdot 9H_2O$	5.21±0.21
40 nM haemin	2.60±0.21
40 nM haemin + 64 nM Zn-ppIX	1.14±0.03
40 nM haemin + 64 nM Zn-ppIX + 2.4 μM $FeSO_4 \cdot 7H_2O$	2.64±0.18
40 nM haemin + 64 nM Zn-ppIX + 1.6 μM $Fe(NO_3)_3 \cdot 9H_2O$	2.85±0.14

DFO, desferrioxamine; Zn-ppIX, zinc-protoporphyrin IX.
[a] All values represent the means derived from cell cultures in triplicate ± standard deviation (S.D.).

One of the degradation products of haemin, carbon monoxide, has also been reported to be a signal molecule related to cGMP production [8,9]. 8-bromoguanosine-3′, 5′-cyclophosphate (8-Br-cGMP) tested at a wide range of concentrations (0.3 μM to 2.4 mM) did not substantially enhance the mitogenic effects of EGF and insulin, but only resulted in a less than 1.5-fold difference in cell growth (data not shown). In contrast, metHb or haemin could potentiate the mitogenic effects of EGF and insulin and increased the cell growth more than 6-fold (Figs. 1 and 6).

4. Discussion

In this study, I searched for evidence to support the idea that haemoglobin originating from extravasated red blood cells and subsequently oxidised to metHb, a condition often found in tumours showing neovascularisation and haemorrhage, contributes to the increased proliferation of cancerous cells. Indeed, I found that metHb substantially increased the growth of three types of transformed human epithelial cells, representing cells at different stages of cancer development (precrisis and immortalisation) (Fig. 1).

Two earlier studies suggested that metHb might release its content of ferrihaem under suitable conditions because globin and ferrihaem are not joined by a covalent bond [13,25]. In experimental conditions identical to those used for cell culture, I found that human metHb released its haemin content into the culture medium (Fig. 5). Later, in a chemically-defined serum-

free medium that eliminated the complications arising from the use of serum for cell cultures, haemin, like human metHb, dramatically enhanced the proliferation of NW-1 cells (Fig. 6). The addition of chicken antibody (Fig. 5) or haptoglobin (Fig. 3), may have strengthened the binding between haemin and globin [6], thereby blocking the haemin release, and reducing human metHb-induced cell growth. Globin has been shown to contain sequences with opioid activity [27] and might also contain sequences with mitogenic activity, which can be realised when it serves as a growth supplement and is subsequently hydrolysed, by a specific proteolytic enzyme secreted by a subset of transformed cells. However, the globin moiety that was prepared for testing precipitated in the culture medium, resulting in the absence of change seen in cell growth (Fig. 1). It is likely that human metHb under the conditions examined in the present study acts through haemin to become growth-stimulatory.

Haemin was previously reported to activate the HO system in different cell types, including human airway epithelial cells [28]. Haemin in the microsomal fraction is reduced to haem by nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) through the complex HO system. With the introduction of more NADPH and oxygen, ferric ion and carbon monoxide are generated from haem, and the rest of the haem molecule is oxidatively cleaved to biliverdin. Haemin was reported to activate particulate GCase [29], and although a number of recent reports have demonstrated that carbon monoxide acts to function through cGMP as a signal molecule [8,9], the possibility of haemin acting initially through cGMP to stimulate the proliferation of NW-1 cells seems unlikely: (a) haemin-induced growth of NW-1 cells, shown in Fig. 8, was not inhibited by ODQ, a selective specific inhibitor of soluble GCase [24]; (b) ppIX possibly activates soluble GCase [21], yet it did not stimulate the cell growth (Fig. 6); and (c) a membrane-permeable and non-degradable cGMP analogue, 8-Br-cGMP, did not increase cell growth (data not shown). In addition, both haemin and zn-ppIX are inhibitors of soluble GCase [29,30]. If the proliferation of NW-1 cells really depended on the availability of cGMP, cultures of cells in the presence of either metalloporphyrin should all exhibit growth inhibition; however, only zn-ppIX showed such an inhibitory effect (Fig. 8), whereas haemin enhanced cell proliferation (Fig. 6). The inhibitory concentrations of zn-ppIX demonstrated here were less than 1 μM, a quantity possibly insufficient to compete with the haem as the integral part of the soluble GCase, thereby inhibiting the enzyme. Instead, Zn-ppIX here likely inhibits HO, as was pointed out previously [31]. The growth stimulation observed here is also not attributable to biliverdin or bilirubin, as some of the doses tested would be stimulating if haemin were used (Figs. 6 and 8), even though both may function as scavengers of peroxyl radicals [7]. Although carbon monoxide might stimulate cell proliferation through cGMP-independent signal pathways, this seems unlikely in view of the fact that ppIX also converts into carbon monoxide, but was not growth-stimulatory (Fig. 6). Haemin and carbon monoxide were previously reported to be responsible for an increase in PGE_2 production and release from hypothalamic explants [10,11]. PGE_2 in turn enhances the growth of U937 human myeloid leukaemic cells [32]. The results shown in Fig. 6 and data not shown indicated that PGE_2 in NW-1 cells was not growth-stimulating. Therefore, it is unlikely that haemin acts through a PGE_2-mediated pathway to enhance cell growth.

High doses of human metHb and haemin tended to exert a growth-inhibitory effect (Figs. 1 and 6), suggesting that catalytically active iron derived from the catabolic degradation of larger amounts of haem might be responsible. When haem is targeted for degradation, nitrosylation of haem a reaction between haem and nitric oxide possibly synthesised in the microsomal membranes of epithelial cells [33], and which possibly sequesters haem from HO-mediated catabolism, probably no longer plays a significant role in the desensitisation of NW-1 cells to oxidative damage, as has been suggested [34]. Data obtained here, on the other hand, suggest that iron derived from haem degradation is unlikely to contribute to the growth-inhibitory effect of metHb or haemin. As shown in Fig. 6, 2.4 μM of haemin completely inhibited cell proliferation, yet Fig. 7 shows that over 10 times that amount of ferrous or ferric ion added to cultures still stimulated growth. Bilirubin, the ultimate catabolic product of haem in human cells, while commonly viewed as an antioxidant, actually inhibited cell proliferation at concentrations of 0.32 μM and higher (Fig. 8). We reason that the increased amount of bilirubin, possibly derived from metHb or haemin, was responsible for the growth retardation of NW-1 cells.

Irons inside tumour cells form an iron pool, which probably remains ill-defined. To adequately cope with increased rates of proliferation, neoplastic cells might develop an unusual machinery to handle a greater amount of irons that are subsequently needed for energy production, DNA synthesis, and other iron-dependent functions. Induction of ferritin is one of the responses needed to reduce the toxic effects of redox-active iron, and ferritin serves as a warehouse where irons are stored and redistributed [35]. A previous study showed that the ferritin concentration, and iron to apoferritin ratio in ferritin, in human hepatoma cells were lower than those in normal liver cells [36]. Another study showed that hepatic hemosiderin was present in only 10% of 40 human haepatomas examined, but was present in 65% of the adjacent non-neoplastic liver tissue [37]. These studies indicate that the cellular uptake of irons in the form of haemin or inorganic iron salts might be shunted

directly to all active sites for immediate utilisation, and thereupon tumour cells possibly divide at a faster rate. These notions require further studies, perhaps using NW-1 cells for confirmatory experiments.

Bilirubin formed in peripheral tissues inside the human body is transported to the liver for further processing and eventually excreted, indicating that bilirubin may never reach a local level toxic enough to inhibit tumour growth. This likelihood, along with consideration of the results obtained from this study, suggests that haemoglobin derived from extravasated red blood cells in tumours showing neovascularisation and haemorrhage may significantly contribute to uncontrolled tumour growth.

Acknowledgements

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