In vitro erythrophagocytosis by renal tubular cells and tubular toxicity by haemoglobin and iron

Neil S. Sheerin, Steven H. Sacks and Giovanni B. Fogazzi

Department of Nephrology and Transplantation, Guy’s Hospital UMDS, London, UK and 1Divisione di Nefrologia e Dialisi, Ospedale Maggiore, IRCCS, Milano, Italy

Abstract

Background. Patients with gross haematuria of glomerular origin may develop acute tubular necrosis and reversible renal failure. Erythrocytes within the cytoplasm of proximal tubular epithelial cells (PTECs) can be seen on examination of renal biopsies from these patients. It is possible, therefore, that the tubular damage is a result of cytotoxic breakdown products released during erythrocyte degradation.

Methods. To test this hypothesis, we evaluated (i) by transmission electron microscopy, the capability of a PTEC line to phagocyte and degrade erythrocytes in vitro; and (ii) the effect on the viability of PTECs in vitro both after erythrocytosis and after incubation with haemoglobin, free iron or both.

Results. Electron microscopic examination of PTECs exposed to erythrocytes for 96 h showed that 22% of PTECs contained one or more erythrocyte. These were within phagolysosomes and showed varying stages of degradation, with collapse and breakdown of the cell membrane and invasion by cytoplasmic organelles (the so-called haemolytic pathway of erythrocyte degradation). Despite the phagocytosis and degradation of the erythrocytes, no cytotoxicity could be demonstrated under the experimental conditions used. However, the presence of haemoglobin, free iron or both in the culture medium was toxic to the PTECs, resulting in a significant reduction in the number of viable cells present.

Conclusions. PTECs are able to phagocyte and degrade erythrocytes, and haemoglobin and iron are toxic to proximal tubular cells in vitro.

Key words: acute tubular necrosis; erythrocyte degradation; erythrophagocytosis; haemoglobin tubular toxicity; iron tubular toxicity; proximal tubular cell culture condition, which has been described in ~50 patients, most of whom had IgA nephropathy [1–10]. At renal biopsy, the glomerular lesions are usually mild, while the tubulo-interstitial changes are severe. These consist of acute tubular necrosis associated with variable amounts of intra-tubular erythrocyte casts, phagocytosis of erythrocytes by proximal tubular cells, interstitial oedema and interstitial haemorrhages. It has been hypothesized that the decline in renal function is caused by intra-tubular obstruction by erythrocyte casts [1,11]. However, the failure to demonstrate retro-diffusion of Tamm–Horsfall protein into the glomeruli of these patients [9] does not support the hypothesis of intra-tubular obstruction. Thus, other mechanisms have been suggested to explain acute tubular necrosis and renal failure, such as direct tubulo-toxicity of haem pigments or intra-renal vasoconstriction from binding of nitric oxide by haem-containing molecules [12].

In this study, we first investigated the capacity of proximal tubular epithelial cells (PTECs) to phagocytose and degrade erythrocytes in vitro. Then, we assessed whether erythrophagocytosis, haemoglobin, free iron or both are toxic to PTECs in culture.

Subjects and methods

General chemicals were purchased from Sigma (Poole, UK). Tissue culture plastics, culture media and supplements were from Life Sciences (Paisley, UK). The human proximal tubular cell clone was provided by Professor L. C. Racusen (Baltimore, MD). The production and characteristics of this clone have been described previously [13].

Morphological studies

A total of 25 × 10³ PTECs in 1 ml of culture medium were added to each well of a 24-well plate, each well containing a sterile glass cover slip coated with 1% gelatin. After 24 h to allow attachment, the cells were incubated for 24, 48, 72 or 96 h in the presence of whole human blood added to the media to provide a final concentration of 5, 25 and 100 × 10³ erythrocytes/ml of media. Controls were grown in the presence of growth medium alone. For light microscopy, cells were fixed in 50% acetone, 50% methanol for 10 min at 4°C,
air dried and stained with Meyers haematoxolin and eosin. For electron microscopy, the cells were fixed in glutaraldehyde, embedded in taoab embedding resin and stained with uranyl acetate and lead citrate. The sections were viewed with a Hitachi H7000 transmission electron microscope.

Cell viability assay

Serial 2-fold dilutions of PTECs were grown in 96-well plates (1.5 x 10^2–2 x 10^4 cells per well) for 24 h to allow attachment. The cells were then incubated in the presence of: (i) whole blood (25 x 10^9 erythrocytes/ml); (ii) washed erythrocytes; or (iii) control medium for 96, 120 and 144 h. After the incubation period, the medium was removed, the cells were washed and 200 µl of fresh medium containing 3 mg/ml of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to each well and incubated for 4 h at 37°C [14]. Metabolism of MTT by the ubiquitous mitochondrial enzyme, hexosaminidase, results in the production of an insoluble product, the quantity of which is proportional to the number of viable cells present. The culture medium was then replaced with 200 µl of dimethyl sulfoxide per well to solubilize intracellular products, followed by 25 µl of Sorensen’s glycine buffer (0.1 M glycine, 0.1 M NaCl equilibrated to pH 10.5 with 0.1 M NaOH). The optical density of the plates was read at 570 nm.

Assay of haemoglobin and iron toxicity

The potential iron toxicity in our cell system was investigated using a modification of the method described by Zager and Foerder [15]. A solution of 2 mM FeSO_4, 2 mM FeCl_3 and 10 mM ADP in DMEM:F12 medium was used as a source of reactive iron. Purified haemoglobin was prepared by the method of Rossi et al. [16], and the concentration was measured from its absorbance at 500, 510, 542 and 577 nm [17]. Haemoglobin was added to the culture media at a final concentration of 20 µg/ml, a concentration within the range seen in haem pigment-induced renal injury [18]. PTECs were grown in medium containing iron, free haemoglobin, iron + haemoglobin (at the same concentrations as when added to PTECs separately) or DMEM:F12 medium as control for 48 h. After this incubation period, cell viability was assessed by the MTT assay as described above.

Statistics

The analysis of variance was used to test the significance in both the cell viability assay and the assay of haemoglobin and iron toxicity.

Results

In vitro incubation of human erythrocytes with PTECs

The preliminary trial with different incubation times and erythrocyte concentrations showed that a possible erythrophagocytosis could be seen by light microscopy at any incubation time from 24 to 96 h and with any erythrocyte concentration. However, the highest number of erythrocytes within the cytoplasm of PTECs was obtained with a concentration of 25 x 10^3 erythrocytes/ml of medium and after an incubation of 96 h (data not shown). Therefore, subsequent experiments were performed under these conditions. By light microscopy, 20/500 (4%) PTECs apparently contained one or more erythrocytes within their cytoplasm. Electron microscopy unequivocally demonstrated that a proportion of PTECs (35/155, 22.5%) incorporated one or more erythrocytes within phagolysosomes (Figure 1). The electron density of the cytoplasm of the erythrocytes was either normal or was reduced with a finely granular appearance (Figure 2). The phagolysosomes frequently contained a membranous structure, presumably the original erythrocyte membrane, which was surrounded by a space of density equal to that of the erythrocyte. This membrane varied in thickness and density, could show breaks and could be either smooth or wrinkled (Figure 3). A second membrane, which separated the phagocytosed erythrocyte from the cytoplasm of the PTEC, could be seen at higher magnification (Figure 4). In addition, some phagolysosomes were indented by cytoplasmic myelolike figures (Figure 5), or showed small cytoplasmic invaginations (Figure 3). Some other phagolysosomes had blebs protruding from their surface.

Toxicity of erythrocytes, haemoglobin and iron

During the degradation of the erythrocytes by the PTECs, haemoglobin should be released into the lysosomes with subsequent breakdown to release free iron. However, when the PTECs were incubated with the whole blood or washed erythrocytes for 96 h, no evidence of cytotoxicity was seen (Figure 6). Increasing the incubation period to 120 and 144 h had no effect on the viability of PTECs (data not shown). We therefore assessed whether haemoglobin and free iron, either separately or combined, would be toxic to this PTEC line in vitro. The presence of either haemoglobin or free iron in the tissue culture supernatant proved toxic to the PTECs used in this study (P < 0.05 for both observations) after 48 h of incubation (Figure 7). The effect of iron and haemoglobin was additive, with the presence of both in the culture medium resulting in significantly greater toxicity than either iron or haemoglobin alone (P < 0.05; P < 0.001 vs control).

We did not perform electron microscope studies on PTECs to evaluate the type of damage caused by haemoglobin and/or iron nor any investigation to evaluate whether the cell death was due to necrosis rather than to apoptosis.

Discussion

Our report confirms previous observations, based on renal biopsies [1,9,19], that human proximal tubular cells can phagocytose erythrocytes. In man, erythrophagocytosis is not restricted to tubules but can also occur in the cells of breast and lung tumours [20–22], as well as in cells of the trabecular meshwork of the eye [23], T-cells [24], Kupffer cells of the liver [25] and bone marrow histiocytes [26]. Moreover, it can be induced in animals under different experimental conditions. In rats, erythrophagocytosis can occur within the liver cells [27,28], peritoneal and spleen macrophages [29–31], adrenal cells [32], bladder cells [33], bone marrow mast cells [34], thyroid cells [35], renal interstitial cells [36] and adipocytes [37].
**Fig. 1.** Two PTECs containing in their cytoplasm one (right) and three (left) erythrocytes with normal haemoglobin density (electron microscopy, × 7500).

**Fig. 2.** A PTEC containing four erythrocytes in different phases of degradation (arrows) as suggested by both decreasing haemoglobin density and shrinkage of the erythrocyte membrane (electron microscopy, × 5000).
Fig. 3. Two erythrocytes within a phagolysosome. The membrane of one erythrocyte has several breaks (arrows) through which haemoglobin has escaped, causing the formation of a space of density equal to that of the erythrocyte. Note the shrinkage of the membrane of the other erythrocyte, and the two cytoplasmic invaginations into the phagolysosome (the so-called tunnels) (arrowheads) (detail of Figure 2, × 30 000).

Fig. 4. The thin membrane (arrows) which separates the phagolysosome from the cytoplasm of the PTEC. A shrunken erythrocyte membrane as well as a space around it can also be seen (electron microscopy, × 100 000).
guinea pigs, erythrophagocytosis was observed within the epidermal cells of the skin [38], in rabbits within the cells of the spleen and liver [39], in sheep within the trophoblastic epithelial cells of placenta [40], and in goats within the cells of the hemal nodes [41].

Interestingly, our *in vitro* findings are similar to many of those described in the above situations *in vivo*. As in other studies [29–33,35,36,39–41], we found that a single cell could incorporate two or more erythrocytes within lysosomes, and that these could be in different phases of degradation, as documented by a reduction of the electron density of the haemoglobin content. Other findings, such as the internal membrane surrounded by a space with the same density as that of the erythrocyte, the cytoplasmic invaginations or myelin-like bodies within the erythrocytes and the dense bodies protruding from the erythrocytes, were also observed *in vivo* [30–33,35,39–41]. Altogether, these changes indicate that after erythrocytes have been phagocytosed by the tubular cells, they undergo a complex process of degradation, which has been called the haemolytic pathway, and differs from a second degradation pathway, the so-called granular pathway [35].

Our electron microscopy findings in human tubular cells are consistent with those described by Madsen *et al.* [42], who studied the intracellular changes occurring after the injection of autologous blood into the lumen of proximal tubules of rats. Moreover, our results confirmed the data reported by Hill *et al.* [19], who performed the only electron microscope study on tubular changes occurring in glomerulonephritic patients with acute tubular necrosis caused by glomerular bleeding. Thus, with our *in vitro* experiment, we provide a further demonstration that erythrophagocytosis by tubular cells occurs, that this process is similar to that observed in several other organs both in humans and animals, and that it is followed by erythrocyte degradation via the haemolytic pathway.

To evaluate if there is any relationship between tubular erythrophagocytosis and renal dysfunction as seen in patients who were described by several authors [1–10], we studied the effect of erythrocytes and their degradation products on PTECs in culture. We were able to demonstrate that free iron and haemoglobin were toxic to PTECs and that this effect was additive. This finding supports previous results of others, who have demonstrated that haem proteins are toxic to renal tubules [43,44].

However, we were unable to demonstrate any toxicity during incubation with erythrocytes, despite evidence that erythrocytes were undergoing degradation, and presumably releasing haemoglobin and iron. This may reflect a true lack of toxicity. The lysosomal degradation of erythrocytes could result in products that, because of their chemical state or sub-cellular localization, are not toxic to the cell. Alternatively, it is possible that erythrocytes are only toxic to tubular cells under particular conditions which differ from those used in this experiment. For example, the tubular damage caused by haem proteins occurs only at acidic pH [44], is multifactorial, also depending on renal vasoconstriction and haem protein cast formation [45].
A possible explanation is that erythrophagocytosis may be toxic to the PTECs but the detection system used was insufficiently sensitive to detect this. Erythrophagocytosis can only be demonstrated in a limited number of cells, even by electron microscopy and, therefore, a low level of cell death may occur which is not detected. If cell death does occur, it may affect other cells within the system, perhaps inducing proliferative responses, again affecting an assay based on absolute cell number. In addition, the assay used detects cell death but not cell dysfunction, which may be equally as important in vivo as cell death.

Our preliminary study does not provide data about the kinetics of erythrocyte uptake and degradation. Factors which affect the rate of erythrocyte uptake and degradation and the disposal of iron-containing products are likely to alter the extent of PTEC damage. Further in vitro studies are necessary for an understanding of the mechanisms involved in the pathophysiology of acute renal dysfunction in association with macroscopic haematuria.

References

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Fig. 6. Under the experimental conditions used, it was not possible to demonstrate tubular toxicity of either whole blood (top) or washed red blood cells (bottom) after an incubation period of 48 h. Serial increments of PTEC cell concentrations were used to ensure that the cells were in the log phase of growth during the course of the experiment. Points represent the mean ± SEM of six observations.

Fig. 7. The viability of PTECs was significantly reduced by incubation with either iron or haemoglobin (P<0.05) for a period of 48 h. This effect was additive, with increased cytototoxicity seen when PTECs were incubated with both iron and haemoglobin together (P<0.05). Data represent mean ± SEM, n=4–6.
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