Enhanced suicidal erythrocyte death in mice carrying a loss-of-function mutation of the adenomatous polyposis coli gene

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Abstract

Loss-of-function mutations in human adenomatous polyposis coli (APC) lead to multiple colonic adenomatous polyps eventually resulting in colonic carcinoma. Similarly, heterozygous mice carrying defective APC (apcMin/+/H11001) suffer from intestinal tumours. The animals further suffer from anaemia, which in theory could result from accelerated eryptosis, a suicidal erythrocyte death triggered by enhanced cytosolic Ca2+ activity and characterized by cell membrane scrambling and cell shrinkage. To explore, whether APC-deficiency enhances eryptosis, we estimated cell membrane scrambling from annexin V binding, cell size from forward scatter and cytosolic ATP utilizing luciferin–luciferase in isolated erythrocytes from apcMin/+/H11001 mice and wild-type mice (apc+/+/H11001). Clearance of circulating erythrocytes was estimated by carboxyfluorescein-diacetate-succinimidyl-ester labelling. As a result, apcMin/+/H11001 mice were anaemic despite reticulocytosis. Cytosolic ATP was significantly lower and annexin V binding significantly higher in apcMin/+/H11001 erythrocytes than in apc+/+/H11001 erythrocytes. Glucose depletion enhanced annexin V binding, an effect significantly more pronounced in apcMin/+/H11001 erythrocytes than in apc+/+/H11001 erythrocytes. Extracellular Ca2+ removal or inhibition of Ca2+ entry with amiloride (1 mM) blunted the increase but did not abrogate the genotype differences of annexin V binding following glucose depletion. Stimulation of Ca2+-entry by treatment with Ca2+-ionophore ionomycin (10 μM) increased annexin V binding, an effect again significantly more pronounced in apcMin/+/H11001 erythrocytes than in apc+/+/H11001 erythrocytes. Following retrieval and injection into the circulation of the same mice, apcMin/+/H11001 erythrocytes were more rapidly cleared from circulating blood than apc+/+/H11001 erythrocytes. Most labelled erythrocytes were trapped in the spleen, which was significantly enlarged in apcMin/+/H11001 mice. The observations point to accelerated eryptosis and subsequent clearance of apcMin/+/H11001 erythrocytes, which contributes to or even accounts for the enhanced erythrocyte turnover, anaemia and splenomegaly in those mice.

Keywords: phosphatidylserine • cell membrane scrambling • calcium • cell volume • eryptosis • APC

Introduction

The APC protein binds the oncogenic protein β-catenin and favours its degradation [1–4]. Lack of APC is followed by accumulation of β-catenin, which enters the nucleus and stimulates the expression of several genes involved in the regulation of cell proliferation [5, 6]. Loss-of-function mutations affecting APC lead to the development of multiple colonic adenomatous polyps, which eventually results in colonic carcinoma [7, 8]. Mice carrying a mutation in the APC gene (apcMin/+/H11001), which leads to truncation of the APC protein at amino acid 850, develop multiple intestinal tumours [9]. Beyond that the mice suffer from enhanced gastric acid secretion [10], hyperaldosteronism and increased blood pressure [11]. Moreover, the animals were shown to suffer from anaemia, a disorder considered to be secondary to blood loss [12].

A blood count of those mice revealed an anaemia despite excessive reticulocyte numbers. The observation points to enhanced erythrocyte turnover, which may, at least in theory, result from enhanced eryptosis, a suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling [13].
Eryptosis is triggered by activation of Ca\(^{2+}\)-permeable cation channels [14–21]. Cytosolic Ca\(^{2+}\) activates Ca\(^{2+}\)-sensitive K\(^+\) channels [22, 23] leading to exit of KCl with osmotically obliged water and thus to cell shrinkage [24]. Enhanced cytotoxic Ca\(^{2+}\) further stimulates scrambling of the erythrocyte membrane with exposure of phosphatidylserine at the cell surface [21, 25–28]. The Ca\(^{2+}\) sensitivity of cell membrane scrambling is increased by ceramide [29]. Eryptotic cells are rapidly phagocytosed and thus cleared from circulating blood [30–32]. Accordingly, accelerated eryptosis may lead to anaemia [13]. On the other hand, eryptosis is a physiological mechanism preventing haemolysis of defective erythrocytes [13].

This study was performed to elucidate whether the anaemia and reticulocytosis in \(\text{apc}^{\text{Min}^-}\) mice is secondary to increased eryptosis. Thus, eryptosis was determined in erythrocytes from \(\text{apc}^{\text{Min}^-}\) mice and from wild-type mice (\(\text{apc}^{+/+}\)).

Materials and methods

Mice

Mice with mutated \(\text{APC (apc}^{\text{Min}^-}\) and wild-type mice (\(\text{apc}^{+/+}\)) were generated by breeding of \(\text{apc}^{\text{Min}^-}\) mice initially obtained from the Jackson Laboratory. The mice (four to eight per experiment, sex-matched, age as indicated) were fed a control diet (C1314; Altromin, Heidenau, Germany) and had access to drinking water \textit{ad libitum}. Unless otherwise stated, 9- to 26-week-old mice were used.

Blood count and reticulocyte estimation

Blood was withdrawn into heparinized capillaries by puncturing the retrobulbar plexus. To this end, the mice were anaesthetized with diethylether (Roth, Karlsruhe, Germany). Anaesthesia was verified by testing the hind limb reflex. Then, 50 l of blood was taken by puncturing the retrobulbar plexus. For all experiments except for the blood count heparin blood was obtained. EDTA blood was analysed using an electronic haematology counter (scil VET abc, Weinheim, Germany). Relative reticulocyte numbers were determined using the Retic-COUNT reagent (BD, Heidelberg, Germany) according to the manufacturer’s instructions.

Incubation, chemicals and solutions

The erythrocytes were isolated by washing two times with Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO\(_4\), 32 HEPES/NaOH (pH 7.4), 5 glucose, 1 CaCl\(_2\). Erythrocytes were incubated \textit{in vitro} at a haematocrit of 0.4% in Ringer solution at 37°C for 8 hrs unless otherwise stated. Where indicated, amiloride or ionomycin (both Sigma-Aldrich, Schnelldorf, Germany) were added, glucose removed or 1 mM CaCl\(_2\) substituted with 4 mM ethylene glycol tetraacetic acid (EGTA; Sigma-Aldrich).

FACS analysis of annexin V-binding and forward scatter

For FACS analysis of annexin V-binding and forward scatter 50 l cell suspensions were washed in Ringer solution containing 5 mM CaCl\(_2\) and then stained with Annexin-V-FITC (1:250 dilution; Immunotools, Friesoythe, Germany) in this solution for 20 min. at 37°C under protection from light. In the stained erythrocyte cell suspensions, forward scatter of the cells was determined, and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS calibur (BD, Heidelberg, Germany).

Measurement of the \textit{in vivo} clearance of fluorescence-labelled erythrocytes

The \textit{in vivo} clearance of fluorescence-labelled erythrocytes was determined as described previously [33]. Briefly, erythrocytes (obtained from 200 l blood) were fluorescence labelled by staining the cells with 5 lM carboxyfluorescein-diacetate-succinimidyl-ester (CFSE; Molecular Probes, Leiden, The Netherlands) in PBS and incubated for 30 min. at 37°C. After washing twice in PBS containing 1% FCS, the pellet was resuspended in Ringer solution (37°C), and 100 l of the CFSE-labelled erythrocytes were injected into the tail vein of the recipient mouse. As indicated, blood was retrieved from the tail veins of the mice, and CFSE-dependent fluorescence intensity of the erythrocytes was measured in FL-1 as described earlier. The percentage of CFSE-positive erythrocytes was calculated in percentage of the total labelled fraction determined 10 min. after injection.

Confocal microscopy and immunofluorescence

For the detection of annexin V-binding and CFSE-dependent fluorescence of erythrocytes in splenic tissue, the mice were deeply anaesthetized with diethylether. Then, they were killed by cervical dislocation. After laparotomy, the spleens of \(\text{apc}^{\text{Min}^-}\) and of \(\text{apc}^{+/+}\) mice were removed, weighed and mechanically homogenized in 1 ml cold PBS. The suspension was then centrifuged at 500 \(\times\) g for 10 min. at 4°C. The cell pellet was resuspended in 200 l cold PBS. Five microlitres of Annexin V-APC (BD) were added, and incubation was carried out for 20 min. at 37°C protected from light. The suspension was then transferred onto a glass slide and mounted with Prolong\textsuperscript{TM} Gold antifade reagent (Invitrogen). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss Microimaging GmbH, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Estimation of intracellular ATP concentration

For determination of erythrocyte ATP, 80 l of erythrocyte pellets were incubated for 12 hrs at 37°C in Ringer solution with or without glucose (final haematocrit 5%). All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of HClO\(_4\) (5%). After centrifugation, an aliquot of the supernatant (400 l) was adjusted to pH 7.7 by addition of saturated KHCO\(_3\) solution. After dilution of the supernatant, the ATP
Results

Blood count and percentage of reticulocytes

A blood count revealed moderate anaemia of the apcMin/+ mice. Erythrocyte count, haemoglobin concentration and haematocrit were significantly smaller in apcMin/+ than in apcMin/+ mice (Table 1). The mean corpuscular volume was, however, significantly increased. According to FACS analysis, the reticulocyte number was significantly higher in apcMin/+ than in apcMin/+ mice at different ages (4, 6, 8 and 12 weeks), pointing to enhanced erythrocyte formation (Table 1).

Table 1 Anaemia in APC-deficient mice

| Parameter          | apc+/+ | apcMin/+ | Normal range |
|--------------------|--------|----------|--------------|
| RBC (10⁶/μl)       | 10.7 ± 0.6 | 5.5 ± 0.1* | 5.0–9.5      |
| HGB (g/dl)         | 16.4 ± 0.7 | 10.0 ± 0.4* | 10.9–16.3    |
| HCT (%)            | 55.2 ± 2.0 | 34.1 ± 1.2* | 38.5–45.1    |
| MCV (fl)           | 51.8 ± 1.8 | 62.3 ± 1.7* | 48.0–56.0    |
| MCHC (g/dl)        | 29.8 ± 0.5 | 29.3 ± 0.5 | 25.9–35.1    |
| MCH (pg)           | 15.5 ± 0.3 | 18.4 ± 0.7* | 11.9–19.0    |
| Reticulocytes (%)  | 4 weeks (age)  | 3.7 ± 0.3 | 7.0 ± 0.7* | 1.0–6.0      |
|                    | 6 weeks (age)  | 5.3 ± 0.4 | 12.6 ± 0.7* |
|                    | 8 weeks (age)  | 5.3 ± 1.3 | 20.7 ± 4.6* |
|                    | 12 weeks (age) | 4.1 ± 0.4 | 17.8 ± 6.0* |

Arithmetic mean ± S.E.M. (n = 4) of erythrocyte count (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) of 15- to 16-week-old APC-deficient mice (apcMin/) and wild-type mice (apc+/+). Reticulocyte number (n = 4) of APC-deficient mice (apcMin/) and wild-type mice (apc+/+) as a function of age. The data are compared to the normal range in mice [67, 68]. * indicates significant differences between genotypes (Mann–Whitney test; P < 0.05).

Statistics

Data are expressed as arithmetic means ± S.E.M., and statistical analysis was made by non-parametric Mann–Whitney test as indicated in the figure legends using GraphPad InStat Version 3.06 (San Diego, CA, USA); n denotes the number of different erythrocyte specimens studied.

Analysis of the spleen and splenic erythrocytes

As evident from Figure 2A and B, the labelled erythrocytes were mainly trapped in the spleen, which was significantly larger in apcMin/+ mice than in apc+/+ mice. A detailed non-quantitative analysis revealed that the number of fluorescent annexin V binding
and thus phosphatidylserine-exposing erythrocytes was higher in the spleens from apc<sup>Min/+</sup> mice than from apc<sup>+/+</sup> mice (control; Fig. 2C). CFSE accumulates in the cytosol, whereas annexin V binds to phosphatidylserine in the cell membrane.

**Phosphatidylserine exposure of apc<sup>+/+</sup> and apc<sup>Min/+</sup> erythrocytes**

In view of the accelerated clearance of circulating erythrocytes in the spleen of apc<sup>Min/+</sup> mice and their enhanced phosphatidylserine exposure at the cell surface, additional experiments were performed to determine annexin V binding of apc<sup>Min/+</sup> erythrocytes and apc<sup>+/+</sup> erythrocytes in FACS analysis. The experiments were performed in the presence and absence of glucose, as energy depletion is known to foster eryptosis [34]. As shown in Figure 3A and B, annexin V binding reflecting phosphatidylserine exposure at the erythrocyte surface was significantly higher in apc<sup>Min/+</sup> erythrocytes than in apc<sup>+/+</sup> erythrocytes following energy depletion.

**Role of Ca<sup>2+</sup> for cell membrane scrambling of apc<sup>+/+</sup> and apc<sup>Min/+</sup> erythrocytes**

As cytosolic Ca<sup>2+</sup> is important for triggering of eryptosis, the Ca<sup>2+</sup> sensitivity of annexin V binding was tested by exposing apc<sup>Min/+</sup> and apc<sup>+/+</sup> erythrocytes to the Ca<sup>2+</sup> ionophore ionomycin (10 μM). As illustrated in Figure 4A, the ionomycin effect on annexin V binding was significantly stronger in apc<sup>Min/+</sup> erythrocytes than in apc<sup>+/+</sup> erythrocytes pointing to higher Ca<sup>2+</sup> sensitivity of apc<sup>Min/+</sup> erythrocytes. To define the role of Ca<sup>2+</sup> entry for the triggering of energy depletion-induced eryptosis, apc<sup>Min/+</sup> and apc<sup>+/+</sup> erythrocytes were incubated with glucose-free Ringer in the presence and absence of amiloride (1 mM), an
V-binding erythrocytes from APC-deficient mice (apcMin/) without (left bars) or with (right bars) 10 mM ionomycin. *, ** indicate significant (P < 0.05, P < 0.01) difference from absence of amiloride, §§ indicates significant (P < 0.001) difference from the presence of glucose (Mann–Whitney test). © 2011 The Authors

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Cytosolic ATP concentration in apc+/+ and apcMin/+ erythrocytes

Glucose deprivation is likely to affect the intracellular ATP content. Hence, the intracellular ATP concentration of erythrocytes
incubated in the presence or absence of glucose for 12 hrs was determined. As shown in Figure 5, glucose depletion indeed decreased the intracellular ATP concentration of erythrocytes from both genotypes. In the presence of glucose, cytosolic ATP content was significantly lower in apcMin/H11001 erythrocytes than in apc/H11001/H11001 erythrocytes. Following glucose depletion cytosolic ATP content tended to be lower in apcMin/H11001 erythrocytes than in apc/H11001/H11001 erythrocytes, a difference, however, not reaching statistical significance (Fig. 5).

**Cell volume of apc+/+ and apcMin/+ erythrocytes**

To depict cell shrinkage, another hallmark of eryptosis, forward scatter of apcMin/+ erythrocytes and apc+/+ erythrocytes was determined in FACS analysis. As shown in Figure 6, the forward scatter was significantly reduced by energy depletion in erythrocytes from both genotypes, an effect not significantly different between apcMin/+ erythrocytes and apc+/+ erythrocytes.

**Discussion**

According to the present observations, heterozygous mice carrying defective APC (apcMin+/+) suffer from mild anaemia with decreased erythrocyte count, haemoglobin concentration and haematocrit. The anaemia occurs despite significantly higher reticulocyte count in apcMin/+ mice, pointing to enhanced formation of new erythrocytes. Accordingly, the anaemia is secondary to enhanced turnover of apcMin/+ erythrocytes, which is further apparent from accelerated in vivo clearance of CFSE-labelled erythrocytes. The erythrocytes are to a large part trapped in the spleen. The splenic accumulation of eryptotic erythrocytes presumably accounts for the splenomegaly of those mice. Conversely, splenomegaly may foster splenic trapping of erythrocytes.

The accelerated clearance of the apcMin/+ erythrocytes is presumably due to enhanced phosphatidylserine exposure at their surface. Phosphatidylserine-exposing cells are trapped by macrophages [35], engulfed and degraded [36]. Phosphatidylserine-exposing erythrocytes are thus rapidly cleared from circulating blood [32]. To the extent that the accelerated loss of circulating erythrocytes is not matched by a similarly enhanced formation of new erythrocytes, the accelerated eryptosis leads to anaemia.

The present observations do not allow safe conclusions as to the mechanism linking APC deficiency to eryptosis. Clearly, erythrocytes from APC-deficient mice are more susceptible to the eryptotic effects of increased cytosolic Ca²⁺ activity, a property unmasked by the enhanced eryptosis of those erythrocytes following treatment with the Ca²⁺ ionophore ionomycin. The Ca²⁺
Eryptosis has been determined in Ringer, indicating that the enhanced eryptosis was a property of the erythrocytes rather than a result from direct effects of plasma components on erythrocyte survival. Moreover, the clearance of CSFE-labelled erythrocytes from apcMin−/− mice is enhanced even in wild-type mice. The present observation could be explained by a role of APC in the maintenance of cytosolic ATP levels and survival of erythrocytes. Alternatively, the erythrocytes have been rendered more vulnerable to eryptotic stimuli by some component in circulating blood prior to the experiments. It is noteworthy that reticulocytosis increases with age of the animals and may at least in part be related to tumour growth. Anaemia is a well-known complication of malignancy [37, 38] including familial adenomatous polyposis [12, 39]. In view of the present observations, tumour anaemia may at least in part be due to enhanced eryptosis followed by accelerated clearance of eryptotic cells from circulating blood. In patients with malignancy, the eryptosis may be further triggered by cytostatic treatment, as several cytotoxic drugs have been shown to stimulate eryptosis [13]. Eryptosis is triggered by a wide variety of further anaemia-causing xenobiotics and endogenous substances [40–47], and accelerated eryptosis has been observed in anaemia of several clinical disorders [13], including iron deficiency [32], phosphate depletion [48], haemolytic uraemic syndrome [49], sepsis [50], malaria [51–54] or Wilson’s disease [55]. It is considered likely that APC deficiency enhances the susceptibility to the eryptotic effect of those xenobiotics, endogenous substances and clinical disorders. In view of the rapid clearance of erythrocytes, the splenomegaly and the profound anaemia despite reticulocytosis in apcMin−/− mice, confounding triggers of eryptosis may be present in the blood of those mice.

Phosphatidylserine-exposing erythrocytes have been shown to adhere to the vascular wall [56–60], and to stimulate blood clotting [56, 61, 62]. Accordingly, excessive eryptosis due to oxidative stress may lead to derangements of microcirculation and enhanced eryptosis has been suggested to participate in the vascular injury of metabolic syndrome [63], a chronic clinical condition consisting of the clustering of cardiovascular risk factors including hypertension, that in humans relates also to colo-rectal cancer, and other forms of malignancies [64]. Intriguingly, the apcMin−/− mice suffer from hyperaldosteronism and hypertension [11], an observation similarly made in APC patients [65]. Oxidative stress has further been shown to be relevant for ageing of stored red blood cells [66].

In conclusion, lack of APC leads to enhanced suicidal eryptocyte death or eryptosis. The effect contributes to the anaemia in APC-deficient mice and presumably in patients carrying a loss-of-function mutation of the gene encoding the APC protein. Future studies may explore whether eryptosis is similarly enhanced in human patients suffering from APC.

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Conflict of interest

The authors state that they have no conflict of interest to disclose.

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