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# Tissue transglutaminase (TG2) facilitates phosphatidylserine exposure and calpain activity in calcium-induced death of erythrocytes

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*Dear Editor*,

During their daily life, erythrocytes are exposed to a variety of stress situations. On average, they pass once a minute through the lung, where they are exposed to oxidative stress. More than once an hour, they travel through kidney medulla, where they face osmotic shock. Erythrocytes are deformed to squeeze through small capillaries. The loss of erythrocyte cell integrity is pathological; rupture releases hemoglobin to extracellular fluid, which may be filtered at the glomerula of the kidney, precipitate in the acid lumen of the tubules, obliterate the tubules and thus lead to renal failure. To avoid these complications, erythrocytes, as any other cells, require a mechanism allowing them to be disposed without release of intracellular components.

Erythrocytes are devoid of mitochondria and nuclei, and were considered unable to undergo apoptosis. However, recently it has been revealed that treatment of erythrocytes with the $Ca^{2+}$ ionophore ionomycin,[1] or their exposure to oxidative or osmotic stress,[2] situations that mimic red blood cell aging, leads to cell shrinkage, cell membrane blebbing and phosphatidylserine (PS) exposure, all typical features of apoptosis in other cell types. As macrophages are equipped with receptors recognizing PS, erythrocytes exposing PS at their cell surface will be rapidly recognized, engulfed and degraded.[3] While the requirement for $Ca^{2+}$ entry in the induction of cell death was shown for all these three cell death forms,[2] only ionomycin-induced cell death was found to be dependent on activation of $\mu$-calpain,[1] a $Ca^{2+}$-dependent protease.

In addition to calpain, erythrocytes express another calcium-dependent enzyme, tissue transglutaminase (TG2).[4] In human red blood cells (RBCs), $Ca^{2+}$-induced TG2 activation results in crosslinking of membrane skeleton proteins, leading to an irreversible structural fixation of the plasma membrane and of cell shape.[5] TG2 might therefore play an important role in controlling the deformability and fragility, and therefore lifespan of erythrocytes, in a number of pathophysiological situations, accompanied by an increase in free ionic intracellular $Ca^{2+}$ concentration, such as aging, sickle cell or Köln disease. Indeed, in old RBCs, both protein levels and the *in vitro* activity of TG2 are increased.[6] In addition, transglutaminase-catalyzed polymers were isolated from patients with Köln disease[4] or sickle cell anemia[4]-diseases in which the lifespan of RBCs is known to be greatly reduced. Nevertheless, the physiological role of TG2 in the erythrocyte aging process still remains unclear. To address this question, we took advantage of TG2 knockout mice.[7]

The recognition and uptake of dying erythrocytes by macrophages is a sensitive biological measure of cell death. We assessed the rate of phagocytosis of wild-type and TG2-null RBCs by wild-type macrophages, *in vitro*, to explore possible changes in the death program of TG2-null erythrocytes, including those, which might have significance in influencing the *in vivo* clearance. RBCs were induced to die by the $Ca^{2+}$ ionophore ionomycin (1 $\mu$g/ml) for 1 h and were further incubated with wild-type peritoneal macrophages for an additional hour. As shown in Figure 1a, both wild-type and TG2-null RBCs were engulfed by macrophages, even if RBCs were not exposed to ionomycin. This uptake was different from the phagocytosis of ionomycin-treated RBCs, since it was not inhibited by annexin V or by apoptotic Jurkat cells, known competitors of apoptotic cell uptake (data not shown). In contrast, the uptake of ionomycin-treated RBCs above the background, which was fully inhibited by apoptotic Jurkat cells and annexin V, and thus represents uptake of apoptotic RBCs, was significantly reduced in the case of TG2-null RBCs, as compared to the wild types (from $31.2 \pm 3.7\%$ of wild type to $18.7 \pm 2.6\%$ of TG2-null cells, $n = 5$, $P > 0.05$ determined by non-paired *t*-test). These observations suggest that activation of TG2 by $Ca^{2+}$ in dying erythrocytes facilitates recognition by macrophages.

Since the appearance of PS plays a key role in the recognition of RBCs,[3] we investigated the kinetics of PS exposure on the surface of TG2-null RBCs following addition of ionomycin. The exposure of PS was delayed modestly in TG2-null erythrocytes during $Ca^{2+}$-induced death (Figure 1b). However, the absence of TG2 did not affect the cluster formation of PS residues (Figure 1c), which has been shown to be required for proper recognition of apoptotic cells. Since in other cell types TG2 has various biological activities,[4] to make sure that crosslinking activity of TG2 is required for proper

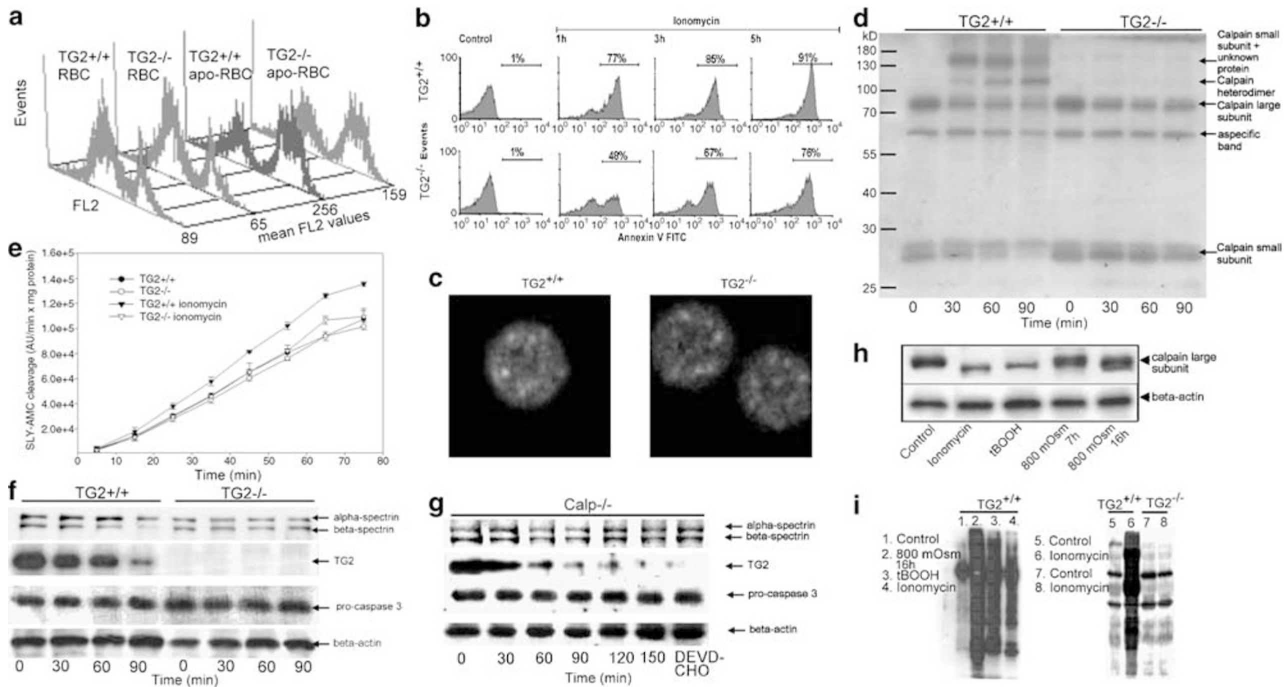

Figure 1 (a–f, h and i) Erythrocytes from adult TG2 $^{+/+}$ and $^{-/-7}$, or (g) from $\mu$-calpain$^{-/-}$mice,[9] were used. Blood was drawn by supra-orbital punctuation, and red blood cells were separated by centrifugation at 1000 r.p.m. for 10 min at 4°C, and maintained in Hank's Buffered Salt Solution. All reagents were purchased from Sigma, except for the ones indicated. (a) Engulfment of ionomycin-treated TG2$^{-/-}$ RBCs by TG2 $^{+/+}$ peritoneal macrophages is delayed as compared to that of TG2 $^{+/+}$ cells. RBCs stained with the fluorescent dye PKH-26 and exposed or not to ionomycin (1 $\mu$g/ml for 1 h) were added to carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR, USA)-labeled macrophages at a ratio of 1 : 10. Following phagocytosis for 1 h, macrophages were analyzed for red fluorescence on Becton-Dickinson FACScan. (b) Externalization of PS on the surface of ionomycin-treated TG2$^{-/-}$ RBCs is delayed as compared to TG2 $^{+/+}$ cells. Annexin V-FITC positivity was determined on BD FACScan after incubating RBCs with ionomycin (1 $\mu$g/ml) for the indicated time periods. (c) Surface distribution of PS induced by ionomycin treatment is not altered in the absence of TG2. For detecting potential changes in PS cluster formation, annexin V-FITC-labeled RBCs were also investigated by a Zeiss LSM 510 confocal microscope. (d) TG2 crosslinks the subunits of $\mu$-calpain during ionomycin-induced apoptosis. RBCs were incubated with ionomycin (1 $\mu$g/ml) for the indicated time periods. Western blot was carried out and membranes were probed with a polyclonal anti-calpain antibody that detects both the large and small subunits (gift from Peter Friedrich, Budapest, Hungary). Proteins in the crosslinked complexes were identified by antibodies specific for the large and small subunits of $\mu$-calpain. (e) Ionomycin pretreatment does not enhance the *in vitro* calpain activity in TG2$^{-/-}$ RBCs. Calpain activity was measured using the *N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC) substrate in the membrane fractions of TG2 $^{+/+}$ and $^{-/-}$ RBCs pretreated or not with ionomycin (1 $\mu$g/ml) for 1 h. Fluorescence of the liberated AMC was monitored in a Wallac Victor[2] multilabel counter for 75 min at 37°C. (f) Ionomycin-induced $\mu$-calpain activity *in vivo* is smaller in TG2$^{-/-}$ RBCs than in the wild types. The time-dependent proteolytic cleavage of certain calpain substrates (spectrin, TG2) and caspase 3 was monitored by Western blot analysis following ionomycin treatment. Membranes were probed with polyclonal anti-spectrin, anti-caspase 3 (Cell Signaling Technology, Lake Placid, NY, USA), polyclonal anti-TG2 (Upstate Biotechnology, Charlottesville, VA, USA) and polyclonal anti-actin antibodies. (g) Spectrin is not degraded in $\mu$-calpain$^{-/-}$ RBCs. Western blot analysis was carried out using samples from $\mu$-calpain$^{-/-}$ RBCs, as indicated above. (h) $\mu$-Calpain is activated in RBCs upon oxidative stress and hyperosmotic shock. Processing of $\mu$-calpain large subunit was monitored by Western blot analysis in ionomycin (1 $\mu$g/ml, 1 h), tert-butyl hydroperoxide (1.5 mM, 1 h)-treated or osmotically stressed (800 mOsm for 7 or 16 h) TG2$^{-/-}$ RBCs. Membranes were probed with polyclonal anti-$\mu$ calpain large subunit domain IV antibody. (i) TG2 is activated in RBCs not only following ionomycin treatment but also upon oxidative stress or hyperosmotic shock. RBCs were kept in the presence of the TG2 substrate biotin cadaverine (Molecular Probes, Eugene, OR, USA) (120 $\mu$M) and the cell death was induced as described in the above section. TG2-dependent incorporation of biotin cadaverine into proteins was detected by Western blot analysis using peroxidase-conjugated streptavidine (GE Healthcare, Bio-Sciences, Hilleroed, Denmark). In comparison, the result of the same Western blot analysis in ionomycin-treated TG2$^{-/-}$ cells is also shown

PS exposure of dying erythrocytes, RBCs were loaded with biotin cadaverine, a competitive inhibitor of TG2 crosslinking activity, and then exposed to ionomycin. The inhibition of TG2 crosslinking activity also delayed the PS exposure of wild-type dying erythrocytes, while it had no effect on the PS exposure of TG2-null cells (data not shown). This suggests that the crosslinking activity of TG2 is required for the observed effect.

To determine which proteins might be crosslinked by TG2 during $Ca^{2+}$-induced apoptosis of erythrocytes, erythrocytes were preincubated with biotin cadaverine. Biotin cadaverine acts as a lysine amine donor substrate for TG2, and following activation, TG2 will incorporate it into its protein substrates. After separation of proteins by 2D gel electrophoresis, the staining for biotin will detect all the biotin-positive proteins, but only those biotinylated proteins will represent true TG2 glutamine donor substrates, which are not labeled in the TG2-null erythrocytes. Some of these Proteins (19) were selected, and their identity was determined by peptide mass fingerprinting (see Supplementary Figure 1S). Among the non-structural proteins, we identified the calpain small subunit 1 as one of TG2 glutamine substrates. Since ionomycin-induced PS exposure was reported to be dependent on $\mu$-calpain,[1] we decided to further investigate the biological consequence of $\mu$-calpain crosslinking by TG2. To confirm that calpain small subunit 1 is indeed a TG2 substrate, the crosslinked forms of it were detected by Western blot analysis using an antibody that detects both the small and the large subunits of $\mu$-calpain. Using specific antibodies for the $\mu$-calpain large and small subunits, we identified the presence of calpain large and small subunits in the various crosslinked

protein complexes (Figure 1d). These data demonstrate that at least one of the proteins to which calpain small subunit 1 is crosslinked by TG2 is itself the $\mu$-calpain large subunit.

Since protein crosslinking by TG2 results in an irreversible modification of calpain structure, if this modification alters calpain activity, it could be detected also after disrupting the RBC structure. Indeed, calpain activity was similar in wild-type and TG2-null cells before addition of ionomycin, but ionomycin treatment enhanced *in vitro* calpain activity only from wild-type cells (Figure 1e). This higher calpain activity in TG2 containing cells during $Ca^{2+}$-induced RBC death could also be demonstrated *in vivo* by detecting the faster cleavage of $\alpha$ and $\beta$ spectrins (Figure 1f), as these proteins are substrates for $\mu$-calpain,[8] and their cleavage during $Ca^{2+}$-induced death is fully dependent on $\mu$-calpain, as demonstrated in $\mu$-calpain-null[9] RBCs (Figure 1g). However, TG2, which is also a $\mu$-calpain substrate,[10] was degraded in $\mu$-calpain-null cells (Figure 1g). Although TG2 is also a substrate for caspase 3, and RBCs express caspase 3, caspase 3 was not activated in those cells (Figure 1g). In addition, DEVD-CHO, an inhibitor of caspase 3, was unable to prevent calcium-induced TG2 cleavage in calpain-null cells (Figure 1g). These data imply that besides $\mu$-calpain, another yet unidentified protease must also be involved in the ionomycin-induced death of erythrocytes.

To test independently whether $\mu$-calpain is indeed involved in enhancing $Ca^{2+}$-induced PS exposure by TG2, we decided to investigate whether TG2 is capable of enhancing PS exposure during death induced by oxidative or osmotic stress; cell death processes reported to be independent of $\mu$-calpain, but dependent on increases in cytosolic $Ca^{2+}$ concentrations.[2] Erythrocytes were exposed to either 1.5 mM tert-butyl hydroperoxide (oxidative stress) or to osmotic (800 mOsm) stress, which induced annexin V positivity in $63.7 \pm 7.8\%$ or $22.4 \pm 3.6\%$ of the wild-type cells, within 1 and 7 h, respectively. In line with the observation that these cases of cell death are dependent on increases of cytosolic $Ca^{2+}$ concentrations,[2] we found proteolytic processing of $\mu$-calpain in both cell death forms detected in $TG2^{-/-}$ cells (to avoid crosslinking of calpain), although with a slower kinetics during osmotic stress (Figure 1h). Detecting *in vivo* activation of TG2 by the incorporation of its artificial substrate biotin cadaverine, we also found activation of TG2 in both cases, but again TG2 was activated very late during osmotic stress (Figure 1i). In accordance with the findings of ionomycin-induced death of RBCs (Figure 1b), as compared to wild-type cells, the PS exposure was delayed in TG2-null cells during osmotic and oxidative stress as well (data not shown). So we decided to reinvestigate the potential role of $\mu$-calpain in regulating PS exposure during calcium-induced death of erythrocytes. In contrast to previous suggestions based on calpain inhibitory studies,[1] we found no difference in the kinetics of PS exposure, when wild type and $\mu$-calpain-null erythrocytes were exposed to either ionomycin treatment, oxidative or osmotic stress (data not shown). Altogether these observations suggest that TG2 using its crosslinking activity influences two independent cell death processes during calcium-induced death of RBCs: it facilitates the $\mu$-calpain-dependent proteolytic cleavage by directly activating $\mu$-calpain, and in addition it accelerates the PS exposure, which appears to be independent of $\mu$-calpain.

Do these effects of TG2 affect the longevity of RBCs? To answer this question, we labeled isolated wild-type and $TG2^{-/-}$ RBCs, stained them with PKH-26 kit for 5 min according to the manufacturers' instructions, reinjected them intraperitoneally into 4-4 wild-type mice (to make sure that the deficiency in the uptake of apoptotic cells by TG2-null macrophages[11] does not affect the clearance rate of injected RBCs) and followed the time-dependent disappearance of labeled erythrocytes. Although uptake of the RBCs from the peritoneum was different, TG2-null cells showing a delay, we found no significant difference in the kinetics of the disappearance of labeled $TG2^{+/+}$ and $TG2^{-/-}$ RBCs from the circulation (Supplementary Figure 2S). These findings demonstrate that TG2 in RBCs, once the death program is initiated, accelerates the program and facilitates the clearance of dying cells, but it does not play a determinant role in the initiation of the cell death program, and thus does not influence the longevity of RBCs.

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