Abstract  Emerin is an almost ubiquitous protein which is abnormal in X-linked Emery-Dreifuss muscular dystrophy (EMD), a syndrome characterized by muscle weakness, joint contractures and cardiac arrhythmia. Emerin is localized in the cells at the nuclear rim and its function is still unknown. In some models, emerin has also been described in the cytoplasm; however, its presence outside the nucleus is still matter of debate. We report the presence of emerin in circulating normal human platelets and its absence in platelets from X-linked EMD patients. Since platelets are cytoplasmic fragments derived from megakaryocytes, the presence of emerin in platelets confirms cytoplasmic localization of this protein, probably related to specific functions. We found also that emerin is present in the cytoplasm of megakaryocytes, while it is absent in circulating granulocytes.

Key words  Emerin · Platelets · Cytoskeleton · Emery-Dreifuss muscular dystrophy

Introduction

Emerin is a 254-amino acids protein identified as the STA gene (Xq 28) product [3]. Mutations in the STA gene leading to emerin absence or alteration are responsible for the X-linked form of Emery-Dreifuss muscular dystrophy (EMD), a syndrome characterized by slowly progressive muscle wasting and weakness, contractures (of the elbows, Achilles’ tendons and post-cervical muscles), cardiac arrhythmia and atrial paralysis [10]. The most dangerous feature of EMD is the cardiac conduction defect, since it can lead to heart block and sudden death even in absence of skeletal muscle involvement; moreover, arrhythmia and sudden death have been reported also in female carriers [2, 9–11].

Emerin, which shares some homology with the nuclear lamina-associated protein 2, is reported to be a nuclear membrane protein, and migrates at 34 kDa on SDS-polyacrylamide gel electrophoresis (PAGE) [3, 14, 18, 23]. Emerin localizes to the nuclear rim in several tissues, such as skeletal, cardiac and smooth muscle, and skin, as well oral mucosa cells, as demonstrated by immunofluorescence [18, 19, 22, 23, 27]; a more precise localization to the nuclear lamina was shown by immunogold electron microscopy [7, 29].

An additional cytoplasmic positivity for emerin in some tissues has also been described [23], but this remained a controversial question [18]. In addition, a cytoplasmic localization of emerin has been reported in two different models: cultured cells (cardiomyocytes, HeLa-S3, SaOS-2, MG63) and myocardium. The cytoplasmic emerin appeared to be not associated with specific structures in the cultured cells, where the emerin fraction present in the cytoplasm was solubilized by detergent treatment and high salt extraction, but the nuclear lamina-associated fraction was demonstrated to be insoluble and was found in the final nuclear matrix [7, 29]. In cardiomyocytes a specific localization at the intercalated disks was found using both polyyclonal [7] and two monoclonal antibodies [20]; on the other hand, several other
monoclonal antibodies either gave uncertain results, or showed no emerin fluorescence in these structures [20]. On the basis of the localization to the nuclear lamina, and of its absence in most X-linked EMD patients, a possible structural role, or an involvement in cell signaling, have been suggested as hypotheses for the emerin function [18, 23] and a possible role of emerin in the myocardium intercalated disks has been suggested for the pathogenesis of the heart conduction defect [7]. Nevertheless, the pathophysiology of this peculiar muscular dystrophy, associated with clinically relevant heart arrhythmias, remains to be explained.

The presence of emerin in circulating nucleated blood cells allows clinical diagnoses to be performed by Western blot analysis [19, 22], but immunocytochemistry on blood smears was considered unreliable [22], while Northern blot analysis for emerin mRNA revealed no single bands in leukocytes, and no evidence of splicing variations [30].

We report that normal blood cells smear, when incubated with polyclonal or monoclonal anti-emerin antibodies, contained small, intensely positive particles which were identified as platelets: we have demonstrated that circulating platelets from healthy individuals contain emerin, while platelets obtained from X-linked EMD patients (carrying null mutations) do not. The emerin present in platelets shows the same electrophoretic mobility of the muscular form; no differences are detectable between resting and surface-activated platelets or using different anticoagulants. We also report that cultured megakaryocytes, the platelets precursors, show positive immunoreactivity for emerin both at the nuclear rim and in the cytoplasm. Hypothetical emerin functions in platelets do not appear to be related to coagulation. Moreover, no emerin immunoreactivity was found in circulating granulocytes.

Materials and methods

Peripheral blood was sampled from informed consenting individuals (according to the Helsinki declaration of 1964 for human rights): three healthy donors and four EMD patients carrying null mutations, belonging to two X-linked families. In three patients, from the first family, the diagnosis was established by clinical and laboratory data [21], by linkage analysis [15] and by molecular analysis, which showed a 29-bp deletion in the STA gene at nucleotide 1740, causing a stop at codon 228 [22, 24]. The control muscle sample for the Western blot analysis consisted of a biopsy specimen from a consenting non-dystrophic patient.

Antibodies

The anti-emerin polyclonal antibody was raised in rabbits and affinity purified as described previously [7, 22]; its specificity was assessed by immunoblotting and immunohistochemistry, by the absence of labeling after either antigen preadsorption or antibody substitution with preimmune serum. The monoclonal anti-emerin antibody (NCL) was purchased from Novocastra (UK). Secondary fluorescein-conjugated antibodies, swine anti-rabbit or rabbit anti-mouse (Dako), were diluted 1:100; the colloidal gold conjugated goat anti-rabbit antibodies (GAR 15 and GAR 5, Amersham) were diluted 1:10.

Blood smears

Blood drops obtained by finger puncture were smeared on glass slides, air dried, fixed with cold methanol (−20°C) for 10 min, washed with PBS, preincubated with 3% dried skimmed milk for 1 h, washed with PBS and incubated with primary anti-emerin polyclonal antibody diluted 1:100, or monoclonal antibody diluted 1:20, with TRIS-buffered saline (TBS)-1% bovine serum albumin (BSA) for 1 h at room temperature. Controls were incubated with TBS-BSA, in the absence of primary antibody.

Western blotting and platelet fractionation

Venous blood obtained by venipuncture (6 ml) was collected in EDTA containing glass tubes (Vacutainer, Becton Dickinson), containing 0.34 M EDTA (12 µl/ml blood) and diluted 1:1 with PBS; platelets were separated on Ficoll-Hypaque gradient by centrifugation at 600 g for 20 min; the upper ring containing platelets and lymphocytes was collected, washed with PBS and the lymphocytes were separated by two centrifugations at 50 g for 15 min. The platelets were recovered from the upper half of the supernatant by centrifugation at 2000 g for 20 min. The plasma (final supernatant) was centrifuged twice at 2000 g to eliminate residual platelets and analyzed by Western blotting to exclude the presence of emerin in solution.

The upper part of the red Ficoll band, containing granulocytes, was recovered and washed with PBS, the erythrocytes were lysed with Ortho-mune Lysing Reagent (Ortho Diagnostic Systems) and the nucleated cells were centrifuged at 50 g for 15 min to check the emerin presence in other circulating nucleated cells.

Platelet fractions in the absence of detergents were prepared as described previously [1, 5]. The heavy membrane fraction was obtained in 0.25 M sucrose, 10 mM TRIS-HCl pH 7.6; then the supernatant was centrifuged at 100,000 g for 1 h to isolate two fractions referred to as cytosol and microsomes. The heavy membranes and the microsomes were mixed together to obtain the total membrane fraction.

Platelets, blood cells, platelet cytosol and total membranes were lysed in 150 mM NaCl, 1 mM NaF, 1 mM EGTA, 1 mM PMSF, 0.25% deoxycholate (Sigma), 1% Nonidet-P40 (Sigma), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin. The muscle control sample was lysed as previously described [18].

Detergent-extracted platelet fractions were obtained from Ficoll gradient-purified platelets by addition of 2% Triton X-100, 10 mM EGTA, 100 mM TRIS-HCl, 2 mg/ml leupeptin, and 2 mM PMSF, pH 7.4. The lysates were centrifuged at 15,600 g for 5 min to sediment the actin cytoskeleton and the supernatant was centrifuged at 100,000 g for 3 h to sediment the membrane-associated skeleton [8].

The blood cells, the control muscle and the platelet fractions were resolved by SDS-PAGE (12% polyacrylamide, 40 µg protein per lane) and the proteins were transferred to nitrocellulose membranes (Amersham). The proteins were blocked for 1 h at room temperature with TBS containing 0.05% Tween 20, 2% BSA and 3% dried skimmed milk. Incubation with anti-emerin primary antibodies, diluted 1:1,000 (polyclonal) and 1:250 (monoclonal) with TBS-Tween 20, was performed for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham) according to the Manufacturer’s specifications.

Electron microscopy

Both resting and surface-activated platelets were obtained as follows: platelet-rich-plasma (PRP) was prepared by whole blood