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# Effect of radiographic contrast media (Iodixanol, Iopromide) on the spectrin/actin-network of the membranous cytoskeleton of erythrocytes

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## Abstract

Red blood cells demonstrate a unique ability for repeated large deformation. Under the influence of a variety of agents, shapes other than the discocyte – e.g. stomatocytes or echinocytes - can be observed. Some radiographic agents induce shape changes from discocytic to echinocytic cells. Especially the echinocyte formation is associated with a rigidification of the cells bearing the risk of a hindered capillary passage of the echinocytes. The mechanisms leading to the formation of echinocytes are not well understood assuming that the membrane cytoskeleton is a key player. That is why this examination was focused on the participation of components of the membrane cytoskeleton in the formation of echinocytes and the protrusions accompanying the formation of echinocytes.

Two radiographic contrast media approved for intra-arterial application were used to study echinocyte formation (Iodixanol320; Iopromide370). In the *in vitro* study serious changes in the membrane cytoskeleton were only found in those erythrocytes incubated in plasma supplemented with Iopromide370 (30%v/v). The shape of the spectrin net was completely altered; from the more homogeneous distribution - typical of cells in autologous plasma and also of cells in plasma supplemented with Iodixanol320 – to a distribution of spectrin concentrated in the membrane-near regions with the appearance of spectrin-actin co-localization. Co-localized spectrin with actin was also found around the membranous roots of protrusions which resemble exocytotic processes. In central parts of the cells there was a pronounced dissociation of spectrin and actin; green coloured condensed spectrin bundles

originating from the cell membrane reached up to the root of the protrusions. Separate from this there were also fine long actin fibres passing through the whole cell.

The incubation of erythrocytes in plasma supplemented with Iopromide370 induced rounded bubble-like protrusions from the cell membrane containing almost completely long bundles of actin fibres.

The examination confirmed earlier studies showing that some radiographic contrast media are able to induce echinocyte formation. Furthermore, subcellular mechanisms were revealed explaining the different effects of Iodixanol in comparison to Iopromide.

## **Introduction**

Human erythrocytes circulate in the body for about 120 days and are normally biconcave discocytes with a diameter of  $\sim 7.5 \mu\text{m}$ , a surface area of  $\sim 140 \mu\text{m}^2$  and a volume of  $\sim 100 \mu\text{m}^3$  [13]. The excess surface area of erythrocytes (which gives them the flattened shape), together with the elasticity of their membranes, provides them with the flexibility needed to pass through very small capillaries (as small as 2–3  $\mu\text{m}$  in diameter [16]). The membrane consists of a phospholipid bilayer with embedded membrane proteins and associated with it from the cytoplasmatic side is a network of proteins, the membrane cytoskeleton [6]. Although the architecture of the red cell and its membrane-associated cytoskeletal network is known in detail [6] the factors that determine and control the characteristic biconcave shape or the shape change during echinocyte formation are poorly understood [11, 44]. It appears that an intact membrane-associated skeleton is vital for normal shape, as several hemolytic disorders characterized by loss of biconcave shape, have been linked to defects in the membrane skeleton [5, 35]. In some hemolytic anemias, such as hereditary spherocytosis, the cells not only become spherical but are also extremely fragile, implying that the membrane skeletons also furnish stability and elasticity to the cell [5, 9, 23, 26, 36]. Reinhart et al. reported that echinocytes did not occur in spectrin-deficient knock-out mice [42]. Therefore, it can be assumed that spectrin is a key membrane skeleton element of erythrocytic shape changes. Also the link between the skeleton and the membrane seems to be important for normal shape as scission of linking proteins prevents any shape changes and reduces deformability of red cell membranes [18].

Red blood cells demonstrate a unique ability for repeated large deformation. Under the influence of a variety of agents, shapes other than the discocyte – e.g. stomatocytes or echinocytes - can be observed [44]. Some radiographic agents induce shape changes from

discocytic to echinocytic cells [2, 15, 20, 24, 30, 43]. The echinocyte formation is associated with a rigidification of the cells [8, 18, 28, 45] bearing the risk of a hindered capillary passage of the echinocytes [21, 39, 40, 41, 46] (what could be demonstrated in different intravital studies in patients with coronary artery disease [3, 4, 19, 38]).

Well rounded inward and outward protrusions as well as acute spicules of the membrane are described [44]; possibly hinting to different underlying mechanisms. Moreover, actin-oligomers are found in a position perpendicular to the cell membrane [12, 48] enabling a prolongation of the microfilamentary actin potentially effecting the protrusion of the erythrocyte membrane occurring in echinocyte formation.

That is why it was examined whether and which components of the membrane cytoskeleton participate in the formation of membrane protrusions.

## **Material and Methods**

The purpose of the investigation was to prove whether radiographic contrast media provoke echinocyte deformations via actin and/or spectrin filaments or whether the participation of both filament types is needed.

### *Radiographic contrast media*

Two radiographic contrast media approved for intra-arterial application with varying iodine concentrations were examined: (Iodixanol 320 mg Iodine/ml, GE Healthcare, München, Germany; Iopromide 370 mg Iodine/ml Bayer/Schering, Berlin, Germany).

### *Blood collection*

Venous blood (20 ml) was collected in a standardized manner from the cubital veins of n=6 healthy adults anticoagulated with potassium EDTA according to the Nordkem workshop criteria without using a tourniquet [1]. The samples were stored in sealed polystyrene tubes. Donors were informed and gave written consent.

### *Sample processing*

Immediately after sampling, plasma and erythrocytes were separated by centrifugation (500 g, 5 min). Plasma was harvested and the plasma/radiographic contrast mixtures (RCM) required for resuspension of the erythrocytes were prepared. Iodixanol or Iopromide in the concentration of 30% were added to the plasma. Then, the red blood cells – without the buffy coat - were resuspended in these mixtures and incubated for five minutes at 37°C.

### *Staining of components of the membrane cytoskeleton*

Air dried blood smears of erythrocytes - incubated in different RCM/plasma mixtures (30% Iodixanol or Iopromide respectively v/v) - were fixed in 2% paraformaldehyde. After short

rinsing in isotonic PBS (phosphate buffered saline) at room temperature the samples were transferred into cold acetone (-20 °C) for 2 minutes to render the cell membranes permeable for the antibodies. Components of the membrane cytoskeleton were double stained in consecutive procedures to display the distribution of the components in the cytoskeleton. The components were stained either in red (actin; first antibody: polyclonal rabbit anti human actin (Biozol, Eching, Germany, dilution 1: 100 in PBS), second antibody: affinity purified goat anti rabbit IgG TRITC conjugated (Sigma, St Louis, USA, dilution 1: 30 in PBS)) or in green (spectrin; first antibody: SPTB mouse anti human monoclonal antibody against spectrin  $\beta$ -chain (Biozol, Eching, Germany, dilution 1: 30 in PBS), second antibody: affinity purified FITC-coupled anti mouse IgG (Sigma, St. Louis, USA, dilution 1: 30 in PBS)). In case of a very close proximity of these components there is an overlay of the colours red and green resulting in a colour shift towards a light yellow-green colour. Vice versa, if there should be a dissociation of very close components then there would be a colour shift from light yellow-green to red and green [17, 34].

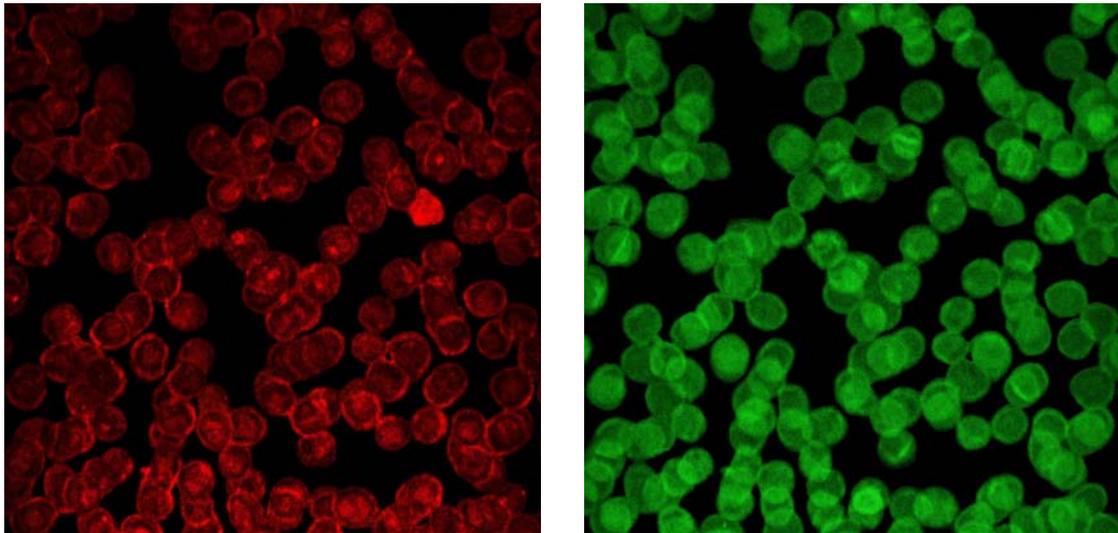
The cells were displayed using confocal laser scanning microscopy at a primary magnification of 1:63 (TCS SP5, Leica, Wetzlar, Germany).

## **Results**

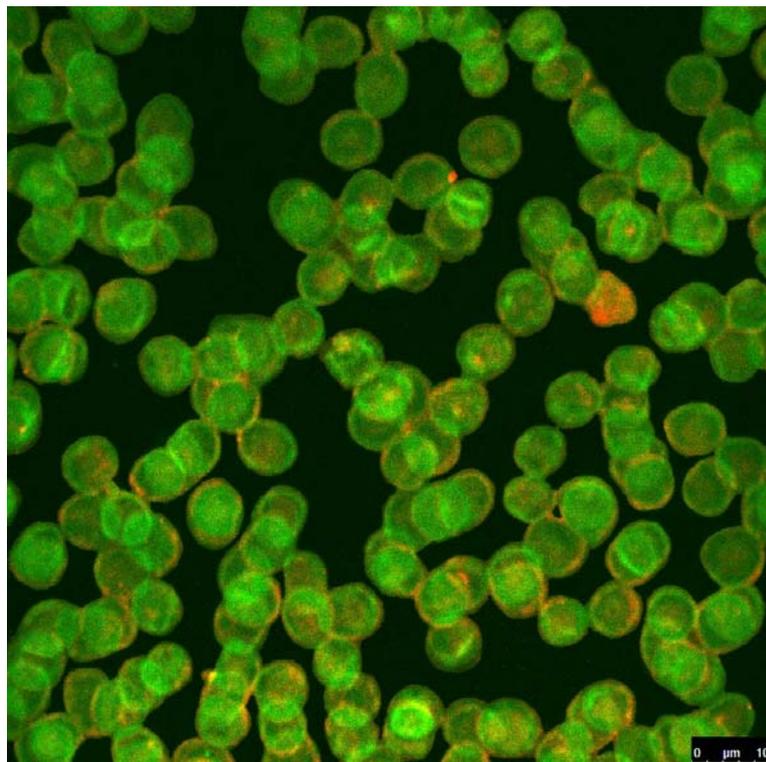
### *Erythrocytes in autologous plasma*

Figure 1 shows erythrocytes in autologous plasma. The membrane cytoskeleton is displayed in a well rounded shape consistent with the shape of discocytes appearing in autologous plasma. The red stained actin component was inhomogeneously distributed over the cells. The actin stain was stronger at the cell periphery and there was a weak stain in the central zone of the erythrocytes. The spectrin stain was spread almost homogeneously over the whole cell.

**Figure 1:** Double stained erythrocytes in autologous plasma (actin: left image in red; spectrin: right image in green)



**Figure 2:** Overlay of the double stained coincident images of Figure 1



In Figure 2 almost no light yellow-green colours are visible showing that spectrin and actin are not close enough or co-localized in discocytes.

Figure 3 shows a magnification of figure 2. Areas where actin and spectrin are co-localized will display light yellow-green colours. The actin display in figure 1 (left image) as well as in figure 3 (white arrow in the figure on the left side) revealed red colours in the centre of the cells. The homogeneous distribution of spectrin in aggregated cells or in clustered cells is displayed in figure 3 as well (white arrow in the figure on the right side). Patches of light yellow-green colours were rarely found and statistically distributed over the cell surface and were very tiny (not greater than one pixel). This indicated that actin and spectrin practically could not be seen to be co-localized in discocytes.

**Figure 3:** Overlay of the double stained (actin, spectrin staining) coincident images of discocytes in autologous plasma

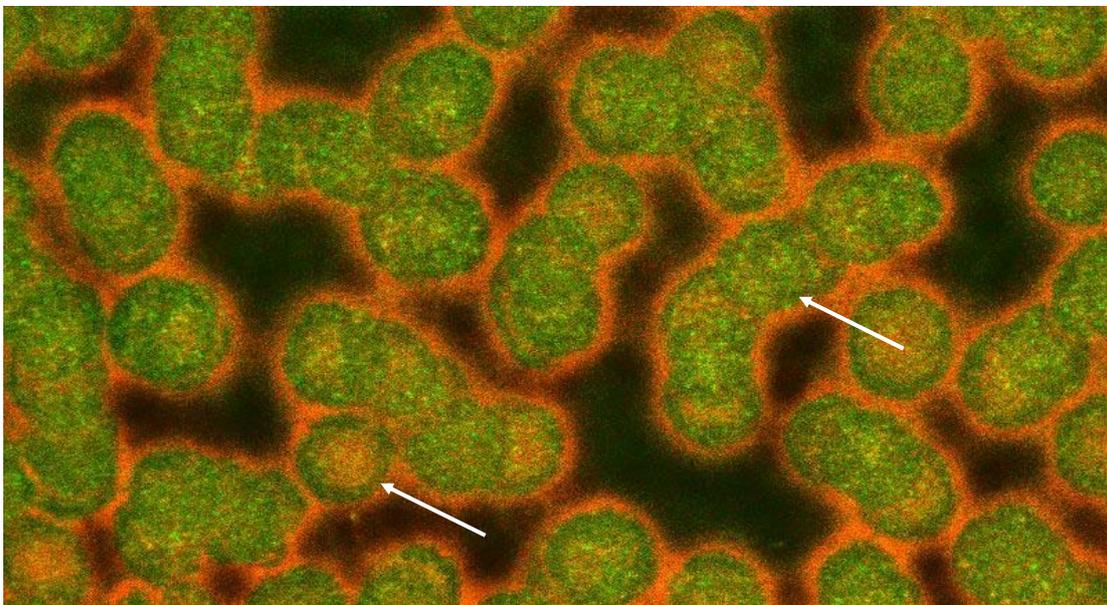


Figure 4 shows magnified details of figure 3 (two erythrocytes marked with white arrows in figure 3). It is evident that actin-spectrin distributions in singularized erythrocytes were different from distributions in aggregated or in clustered erythrocytes. In clustered erythrocytes the spectrin distribution appeared to be more or less homogeneous over the whole cell (see Fig. 4, right), whereas in singularized erythrocytes the red stained actin was displayed in central parts of the cells (see Fig. 4, left).

**Figure 4:** Actin/spectrin distributions in a singularized (left image) or in clustered discocytes (right image)

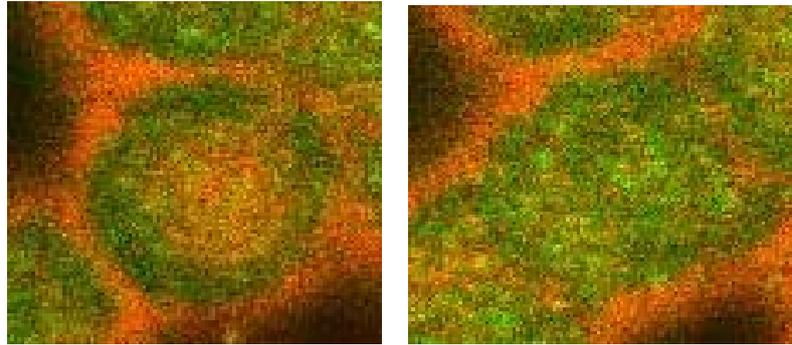
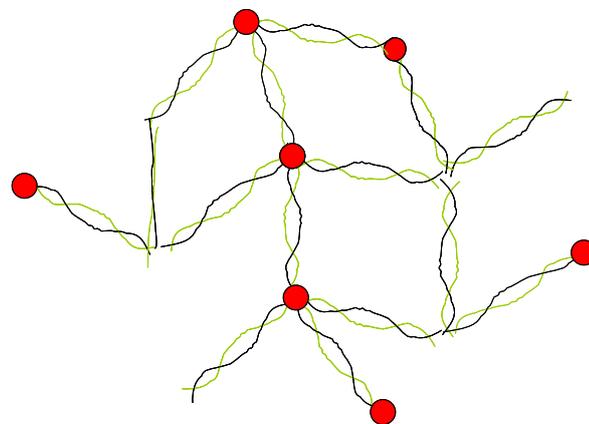
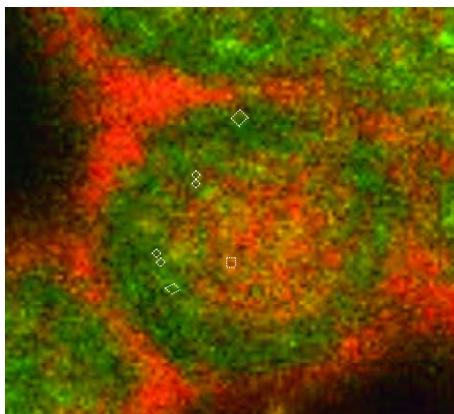


Figure 5 (right image) shows a sketch of the tetrameric spectrin distribution in the membrane skeleton of erythrocytes as published by Drenckhahn and the team of Bennett [12, 25]. It was assumed that a mesh of  $\alpha$ -,  $\beta$ -spectrin tetramers underlies the plasma membrane of erythrocytes linked to the membrane by binding proteins such as ankyrin and band 4.1 [12]. The bond length of the lattice on an average amounted to 100 nm which is of the same order as described for the triangular lattice by Zeman et al. [49] (see figure 5, left). Rectangular structures which were visible after spectrin staining were found all over the erythrocyte surface (see Fig. 5, left), some of them marked through an overlay by dotted white lines.

**Figure 5:** Spectrin-actin-mesh in a discocyte after spectrin-actin double staining displayed by confocal laser scanning microscopy (left image) and a spectrin-actin model of the erythrocytic membrane cytoskeleton (right image, according to [12, 27, 49]) (● : actin-oligomers;  :  $\alpha$ -,  $\beta$ -spectrin tetramers)

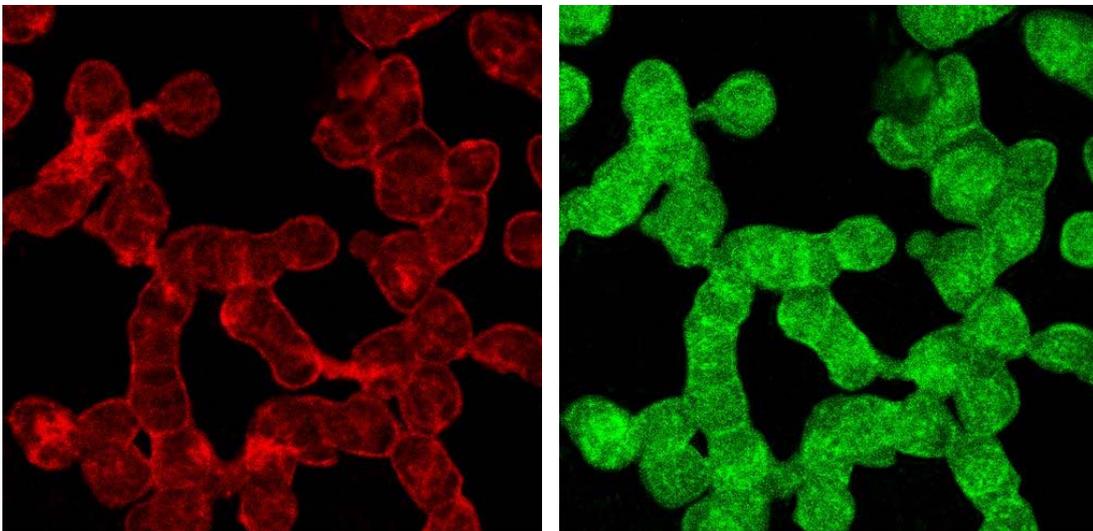


The model of the membrane skeleton foresaw lattice crosspoints with actin oligomers perpendicular to the membrane. The small size of these oligomers (13 actin monomers [49]) could explain why the display of actin is only weak in the erythrocyte magnified in Fig. 5, left.

#### *Erythrocytes in a plasma-Iodixanol-mixture*

Figure 6 shows erythrocytes suspended in plasma supplemented with Iodixanol320 (30% Iodixanol v/v).

**Figure 6:** Double staining of erythrocytes (actin: left image in red; spectrin: right image in green) after incubation in plasma supplemented with Iodixanol320

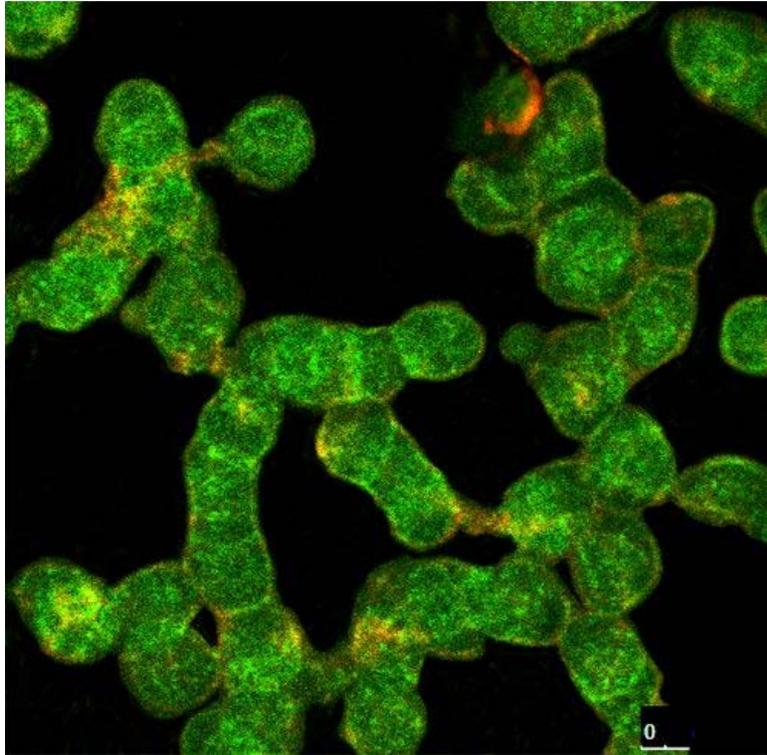


The distribution of actin was inhomogeneous as well with a slight attenuation of actin staining in central parts of the cells compared to cells suspended in autologous plasma. There was a slight enhancement of red stained dots hinting to aggregated actin. A few of these dots showed also in erythrocytes suspended in autologous plasma but there were clearly more of them in erythrocytes suspended in plasma supplemented with Iodixanol. Spectrin stained erythrocytes after incubation in plasma supplemented with Iodixanol did not differ from erythrocytes incubated in autologous plasma.

The overlay of the double stained (actin, spectrin staining) coincident images (Fig. 7) practically did not reveal differences between erythrocytes suspended in autologous plasma or in a plasma/Iodixanol mixture.

It is noteworthy that there was an enhancement of erythrocyte aggregation compared to erythrocytes suspended in autologous plasma.

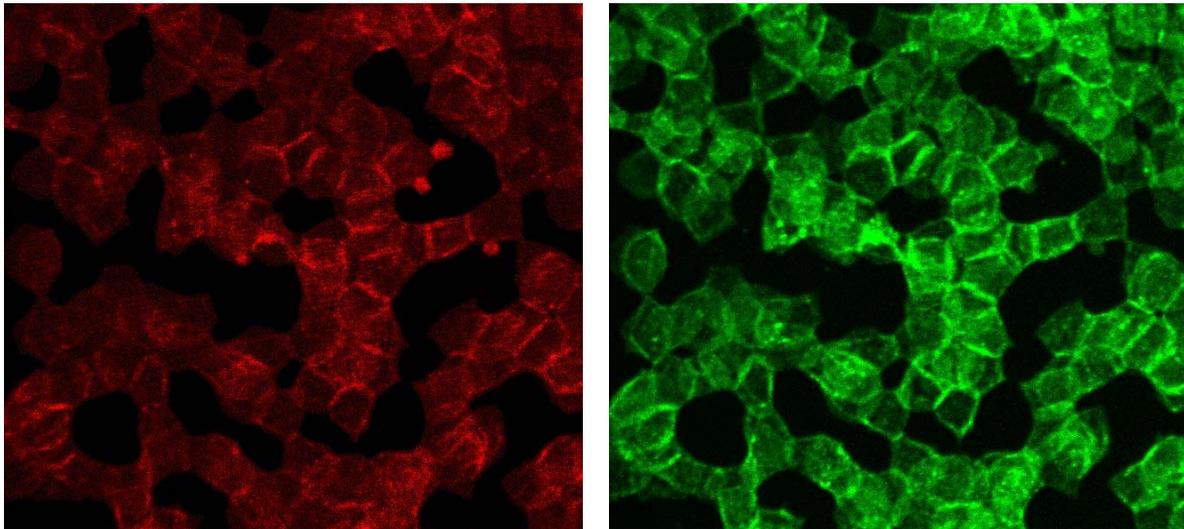
**Figure 7:** Overlay of the double stained (actin, spectrin staining) coincident images of discocytes in plasma supplemented with Iodixanol320 (30% v/v)



*Erythrocytes in a plasma-Iopromide370 mixture*

Figure 8 shows erythrocytes suspended in a plasma supplemented with Iopromide370 (30% Iopromide v/v).

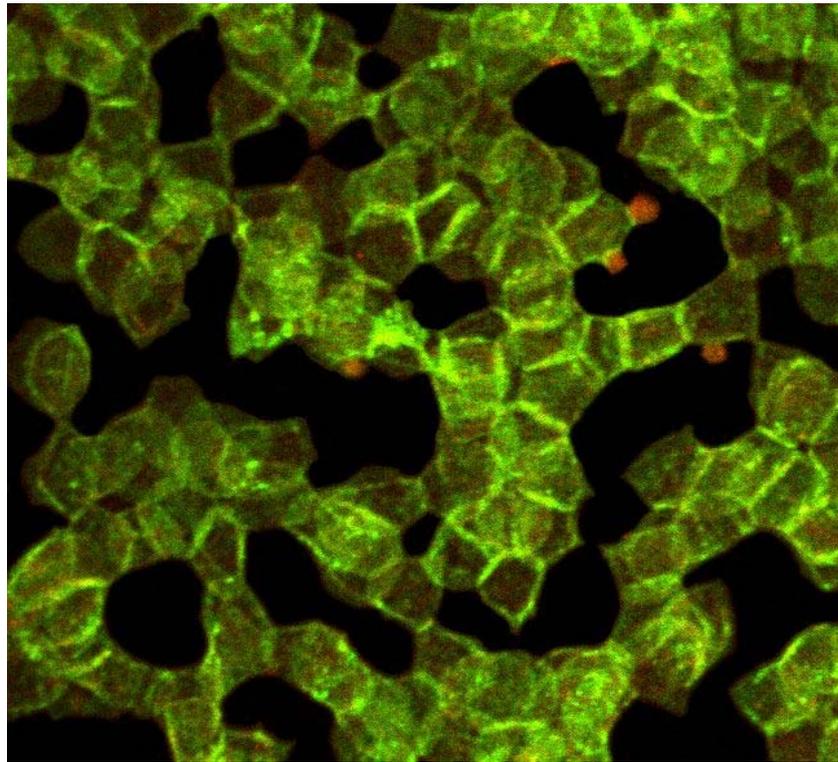
**Figure 8:** Double staining of erythrocytes after incubation in plasma supplemented with Iopromide370 (actin: left image in red; spectrin: right image in green)



The spectrin staining revealed that the membrane cytoskeleton practically acquired a rectangular shape after incubation in plasma supplemented with Iopromide370. This was completely different from the results seen after incubation in a plasma/Iodixanol320 mixture. (One should keep in mind that here the membrane cytoskeleton was displayed and not the shape of the erythrocyte membrane as in the light microscope). This coincided with a completely different distribution of the spectrin mesh. There was no longer the more or less homogeneous spectrin distribution as it appeared after incubation of erythrocytes in autologous plasma or in a plasma/Iodixanol320 mixture. Now, there was a strong spectrin staining at the border of the cells showing also a strong spectrin banding at the cell border and a less strong banding inside the cells (see Fig. 9).

After incubation of erythrocytes in a plasma/Iodixanol320 mixture a slightly enhanced erythrocyte aggregation was observed. Now, in erythrocytes suspended in a plasma/Iopromide370 mixture, there was a strong aggregation of erythrocytes accompanied by rosette formation and by formation of rouleaux and crossed aggregates.

**Figure 9:** Overlay of the double stained (actin in red, spectrin in green) coincident images of erythrocytes in plasma supplemented with Iopromide 370 (30% Iopromide v/v)



This image overlay demonstrates that on one hand there was a clear dissociation of red and green stained components in central cell parts and on the other hand there was a marked association of spectrin and actin components at the cell rim coinciding with a spectrin-actin co-localization revealed by the appearance of light yellow-green colour bands.

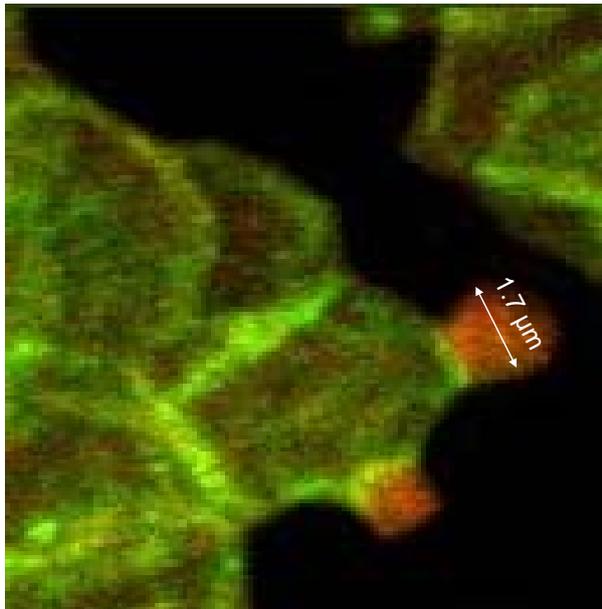
The supplementation of Iopromide370 to the plasma induced changes of the erythrocyte membrane which can be described as rounded bubble-like protrusions. Acuate spicules - as described in literature for echinocytes (see Fig. 10B, modified from [43]) - could not be observed here (though about 80% of erythrocytes show such spicules after incubation of erythrocytes in a Iopromide/plasma mixture [20]). Figure 10A shows erythrocytes after incubation in plasma supplemented with Iopromide370 (confocal laser scanning microscopy, electronically magnified). The actin-spectrin double staining revealed that the well rounded protrusions were at least coated with actin. The rectangularly shaped membrane cytoskeleton mainly showed spectrin and co-localized actin which can be deduced from the light yellow-green coloured bands. Such co-localized spectrin-actin bands were visible also at the membrane roots of the protrusions.

**Figure 10:** Erythrocytes after incubation in a plasma/Iopromide370 mixture (30% v/v)

10 A: confocal laser scanning microscopy

10B: scanning electron microscopy, modified from [43], copyright 2005, with permission from IOS Press.

A



B



Inside the cells there was band like clustering of spectrin, stained weaker, starting from the cell border and oriented towards the membrane roots of the protrusions. The homogeneous distribution of spectrin observed in discocytes could not be demonstrated in central cell parts of echinocytes (see Fig. 10A) where the dissociation of actin and spectrin components was realized instead. This was accompanied by long actin filaments originating from the lateral cell border, passing through whole cells and obviously ending in the opposite cell border. Thick bundles (diameter: 1.7  $\mu\text{m}$ ) of long actin fibres coated (or filled up completely) the protrusions (see Fig. 10A). At the membrane root of the protrusions circular spectrin-actin bands were found whose light yellow-green colours indicated co-localized spectrin and actin.

### Discussion

Here is shown for the first time how the spectrin-actin structure of erythrocytes is changed after their incubation in mixtures of plasma and radiographic contrast media. There was an obvious and strong translocation of spectrin-actin components to the periphery of erythrocytes after suspension in a plasma/Iopromide370 mixture. The co-localization of these two

components - which was rarely observed in discocytes (see Fig. 1 – 4) – leaves us to assume that there was an induction of actin-spectrin complexes at the cell periphery but not in the cell interior. Here a dissociation of long weakly stained spectrin bands from very long and thin actin fibres prevailed (see Fig. 10). These changes were not observed when erythrocytes were incubated in a plasma/Iodixanol320 mixture.

In former publications the incorporation/intercalation of contrast media molecules into the outer leaflet of the membrane bilayer was discussed as the possible cause [20, 43] according to the bilayer couple hypothesis of Sheetz and Singer [37]. This scenario was recently shown to be not very likely [14], so that there is still the question which could be the mechanisms for the echinocyte formation. As a possible and known mechanism for the translocation of parts of the membrane to the exterior or to the interior, e.g. interactions of actin with myosin might be the most likely ones producing movement or inducing stress e.g. through sliding of opposing filaments as is typical of muscle movement. This mechanism seems to be appropriate for exocytotic processes in somatic cells [32] concerning e.g. Weibel-Palade bodies (from endothelial cells [33]), lamellar bodies (from lung cells [29]), or acinar granules (from pancreatic cells [31]), and it might be active also in erythrocytes with the expulsion of the nucleus from pro-erythrocytes, as was shown by the bundling of actin [22].

Supplementation of plasma with Iopromide370, only, led to gross changes of the spatial structure of the membrane cytoskeleton in this study. The homogeneous net-like structure of spectrin – as was found in erythrocytes suspended in autologous plasma or also in plasma supplemented with Iodixanol320 – was changed to a distribution of spectrin concentrated in the membrane-near regions with the appearance of spectrin-actin co-localization coinciding with a transition from discocytes to echinocytes. Co-localized spectrin with actin was also found around the membranous roots of the protrusions. In central parts of the cells there was a pronounced dissociation of spectrin and actin; green coloured condensed spectrin bundles originating from the cell membrane reached up to the root of the protrusions. Separate from this there were also fine long actin filaments passing through the whole cell.

The membrane protrusions appeared as well rounded bubbles (see Fig. 10B) and not as acute spicules (often described for echinocytes). The protrusions were at least coated with dense, very long actin bundles (see Fig. 10A). Spectrin-actin complexes (deduced from the light yellow-green colours possibly indicating the formation of spectrin-actin complexes), were found at the roots of the protrusions and reported to accelerate the actin polymerization [7], which could then generate such long and thin actin fibres observed in this study. The

generation of membrane-anchored actin rings is assumed to support contractile forces in different cell types to effect exocytotic processes [32].

It can be assumed that spectrin bands – found in the cell interior – can act as control structures for the active translocation of actin bundles to the exterior thereby generating a protrusion. The spectrin-actin band at the root of the protrusion could then be the anchoring structure responsible for the relative movement of a membrane part to the outside. The anchoring structure itself seems to be linked locally by membrane proteins (e.g. ankyrin, band 4.1, band 3 [10, 35]) to the surrounding membrane and transcellularly by spectrin bands to lateral and opposite parts of the echinocyte membrane.

At the same time the spectrin bands restrict the deformability of the echinocytes due to a fixation of opposing parts of the erythrocyte membrane. The lowered deformability of echinocytes has been shown multiply in the past [18, 28, 47].

The aggregation of erythrocytes (see Fig. 7) after incubation in plasma/Iopromide370 mixture differed strongly from the behaviour of erythrocytes incubated in plasma/Iodixanol320 mixtures or of cells in autologous plasma in line with earlier studies [2, 15, 20, 24, 43].

The changes in membrane cytoskeleton after addition of radiographic contrast media to the suspension medium were unknown up to now as was the local distribution of cytoskeletal components. Whether the observed alterations are fully reversible has to be doubted. It is especially doubtful whether and how those protrusions filled up with great amounts of actin fibers could be completely relocated. This could coincide with a loss of actin (sometimes found isolated outside of cells) and a possible deterioration of the cell function.

## **Conclusion**

Differences observed in the formation of echinocytes after incubation of erythrocytes in different plasma – radiographic contrast mixtures could be confirmed by this study. Subcellular mechanisms were found as causes for the different effects of Iodixanol320 in comparison to Iopromide370 on echinocyte formation. Evidently Iopromide370 induced stronger and different changes in the membrane cytoskeleton of erythrocytes and echinocyte formation.

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