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Radiographic contrast media alterate the localization of actin/band4.9 in the membrane cytoskeleton of human erythrocytes

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Abstract

Different radiographic contrast media (RCM) were shown to induce morphological changes of blood cells (e.g. erythrocytes or thrombocytes) and endothelial cells. The echinocytic shape change of erythrocytes, particularly, affords alterations of the membrane cytoskeleton.

The cytoskeleton plays a crucial role for the shape and deformability of the red blood cell. Disruption of the interaction between components of the red blood cell membrane cytoskeleton may cause a loss of structural and functional integrity of the membrane.

In this study band4.9 and actin as components of the cytoskeletal junctional complex were examined in human erythrocytes after suspension in autologous plasma or in plasma RCM mixtures (30% v/v Iodixanol-320 or Iopromide-370) followed by a successive double staining with TRITC-/FITC-coupled monoclonal antibodies.

After adding Iopromide-370 to the plasma in practically none of the cells the rounded conformation of the membrane cytoskeleton – as it appeared in cells suspended in autologous plasma – was found. In addition, Iopromide-370 induced thin lines and coarse knob-like structures of band4.9 at the cell periphery while most cell centers were devoid of band4.9, and a box-like arrangement of bands of band4.9. A dissociation between colours red (actin) and green (band4.9) occurred as well. In contrast, erythrocytes suspended in a plasma/Iodixanol-320 mixture showed a membrane cytoskeleton comparable to cells suspended in autologous plasma. Similar results were found with respect to the distribution of actin.

This study revealed for the first time RCM-dependent differences in band4.9 activities as possible pathophysiological mechanism for the chemotoxicity of radiographic contrast media.

Keywords: Radiographic contrast media, band4.9, Iodixanol-320, Iopromide-370, actin, chemotoxicity, cytoskeletal junctional complex, erythrocyte

1. Introduction

Radiographic contrast media (RCM) are widely used to visualize blood vessels. Under the influence of a variety of agents (e.g. RCM), red blood cell shapes other than the discocyte – e.g. stomatocytes or echinocytes - can be observed [14]. Some

RCM induce severe shape changes from discocytic to echinocytic cells [2,20,26,27,33,34,40,48] associated with a rigidification of the cells [8,24,37,52]. The rigidification of echinocytes was reported to bear the risk of a hindered capillary passage up to some minutes in the microcirculation [5,16,32,46,47,53-55] and up to 2 hours in the kidney macrocirculation [54], demonstrated in different *in vivo* studies in patients with coronary artery disease [4,5,25,50]. It was shown in patients with coronary artery disease that the injection of Iopromide-370 into the *A. axillaris* was followed by a pronounced decrease of the erythrocyte velocity (more than 50%) in downstream cutaneous capillaries and in 3 out of 20 patients to a complete standstill over more than 3 minutes [5]. In addition, it was demonstrated that Iopromide-370 application induced a significantly greater drop of the tissue oxygen tension in the myocardium of pigs (from 40.3±10.9 to 22.5±8.9 mmHg; p=0.0003) than the application of Iodixanol-320 (from 42.2±5.6 to 40.7±5.9 mmHg; p=0.0357) [25,36].

The mechanisms effective in the important shape change of erythrocytes are not clear. Well-rounded and larger invaginations appear in the cell membrane resembling the formation of caveolae in some cells as well as slender, narrow and very pointed protrusions of the cell membrane to the outside. These big differences in the shape change of erythrocytes are possibly due to different effective mechanisms.

The membrane bilayer together with the membrane-associated proteins binding the cytoskeleton were described to regulate the characteristic shape, the membrane stability as well as the elastic properties of erythrocytes [38]. Key elements of the cytoskeleton are assumed to be e.g. spectrin, actin, adducin, band4.1 and band4.9

(recently denominated dematin [15]). While the functions of spectrin, actin and band4.1 have been extensively characterized [7,18,35], studies about physiological and pathophysiological functions of band4.9 in erythrocytes are scarce [29,49]. Band4.9 is a component of the junctional complex shown to anchor the tail-end of spectrin to the glucose transporter 1 (GLUT1) in the erythrocyte membrane [12,28,29], - located at the vertices of the mostly hexagonal spectrin filament network [15]. In addition, band4.9 is a potent actin bundling protein *in vitro* and probably also *in vivo* [10] where actin-binding sites were assumed to be contained in the headpiece domain and the intrinsically disordered core domain of band4.9 [10,45]. Very recently Chen et al. gave evidence that band4.9 is most probably monomeric and not trimeric as was assumed before [10].

Holdstock and Ralston were the first who described a membrane stabilizing role for band4.9 [21]. Later on, Khanna et al. reported that the lack of band4.9 headpiece in knock-out mice led to a fragile red cell phenotype with spheroidal erythrocytes, indicating an important role in maintaining the stability of erythrocytes with a marked reduction of their deformability [29].

Thereafter, Lalle et al. demonstrated a more or less homogeneous distribution of knob-like band4.9 stained structures in mouse erythrocytes [31]. Whether the distribution of band4.9 in human erythrocytes is comparable to mouse erythrocytes is unclear up to now, particularly, because band4.9 binds to GLUT1 which is completely lacking in adult mouse erythrocytes [22]. Band4.9 knock-out mice exhibited course, wiry hair and an absence of eyebrows [7,35]. In humans, an

autosomal dominant disease (Marie Unna hereditary hypotrichosis [39,51]), displayed a very similar phenotype and resulted from disruption of 8p21, the genetic locus of band4.9.

Since band4.9 as constituent of the membrane associated protein network was shown to regulate both, the characteristic shape and elastic properties of red blood cells [13], we focused on band4.9 in order to assess whether it was involved in the shape change of blood cells occurring after their interaction with radiographic contrast media.

2. Material and Methods

The work described within this manuscript was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and Uniform Requirements for manuscripts submitted to Biomedical journals as reflected in a priori approval by the institutional review committee of the Medical Faculty of the Heinrich Heine University Düsseldorf (registry number 3522).

The purpose of the investigation was to examine whether contact with RCM provoked a reorganisation of band4.9 and/or actin, which are neighbours and interlinked in the junctional complex of the human erythrocytic membrane cytoskeleton. This was analyzed applying a co-localization technique based on immuno-cytochemical double staining of erythrocytes. Closely spaced elements like actin (stained red) or band4.9 (stained green) will undergo a colour shift from red / green to yellow when these two elements further approach and become co-localized.

2.1 Radiographic contrast media

Two commercially available radiographic contrast media with variant Iodine concentrations and approved for intra-arterial application were examined: Iodixanol

(320 mg Iodine/ml, GE Healthcare, München, Germany) and Iopromide (370 mg Iodine/ml, Bayer/Schering, Berlin, Germany).

2.2 Blood collection

Venous blood (20 ml) was collected from the cubital veins of n = 6 apparently healthy adults in a standardized manner and anticoagulated with potassium EDTA and stored in sealed polystyrene tubes. Biological specimens and all data obtained from their use for research were anonymized.

2.3 Sample processing

Immediately after sampling, plasma and erythrocytes were separated by centrifugation (500 *g*, 5 min). After centrifugation, plasma and buffy coats were removed and erythrocytes were pipetted out of the central region of the packed erythrocytes and then resuspended in autologous plasma (not in a buffer solution). The plasma/radiographic contrast media mixtures required for suspension of the erythrocytes were prepared by adding Iodixanol-320 or Iopromide-370 (30% v/v) to the plasma. Then, the red blood cells - without the buffy coat - were suspended in autologous plasma or these mixtures and incubated for five minutes at 37°C.

The complete examination from blood taking up to finalization of blood smears took less than 2 hours, everything at room temperature and without agitation. A homogenization of the resuspended erythrocytes was performed before the preparation of the blood smears.

2.4 Staining of components of the membrane cytoskeleton

After the incubation of erythrocytes in autologous plasma or in different RCM/plasma mixtures (30% v/v Iodixanol-320 or Iopromide-370, respectively), conventional blood smears were prepared on glass substrates and air dried for two days. For each of the 3 groups 18 slides (3 from every donor) were layered with cells. In the follow up the air dried samples were postfixed in 2% paraformaldehyde for 15

minutes. After short rinsing in isotonic PBS (phosphate buffered saline) at room temperature the samples were transferred into cold acetone (-20 ^{0}C) for 2 minutes to render the cell membranes permeable for the antibodies.

Components of the membrane cytoskeleton were double stained [30] in consecutive steps to display the distribution of these components. The components were stained either in green (band4.9; first antibody: mouse anti human band4.9 polyclonal antibody IgG1 [Biozol, Eching, Germany; dilution 1: 100 in PBS], second antibody: sheep anti mouse IgG FITC conjugated [Sigma, St. Louis, USA; dilution 1 : 100 in PBS]) or in red (actin; first antibody: rabbit anti human-beta actin IgG [Biozol, Eching, Germany; dilution 1: 100 in PBS], second antibody: anti rabbit IgG TRITC conjugated [Sigma, St. Louis, USA; dilution 1: 30 in PBS]).

In case of a very close proximity of these components, confocal laser scanning microscopy allows to visualize a merger of the colours red and green resulting in a colour shift towards yellow colour tones. Vice versa, if there should be a dissociation of earlier closely spaced components then there would be a colour shift from yellow coloration to red and/or green [23,43]. The cells were visualized using confocal laser scanning microscopy at a primary magnification of 1:63 (TCS SP5, Leica, Wetzlar, Germany).

3. Results

Figure 1 shows double stained erythrocytes suspended in autologous plasma in the red (actin) or the green (band4.9) channel of the confocal laser scanning microscope.

Figure 2 shows all band4.9-stained structures. At this higher magnification a knob-like structure and a not quite homogenous distribution of band4.9 in the erythrocytes was found. In most cells, a weakly-stained ring-like area near the rim of the cells appeared surrounded by a peripheral narrow green-stained band.

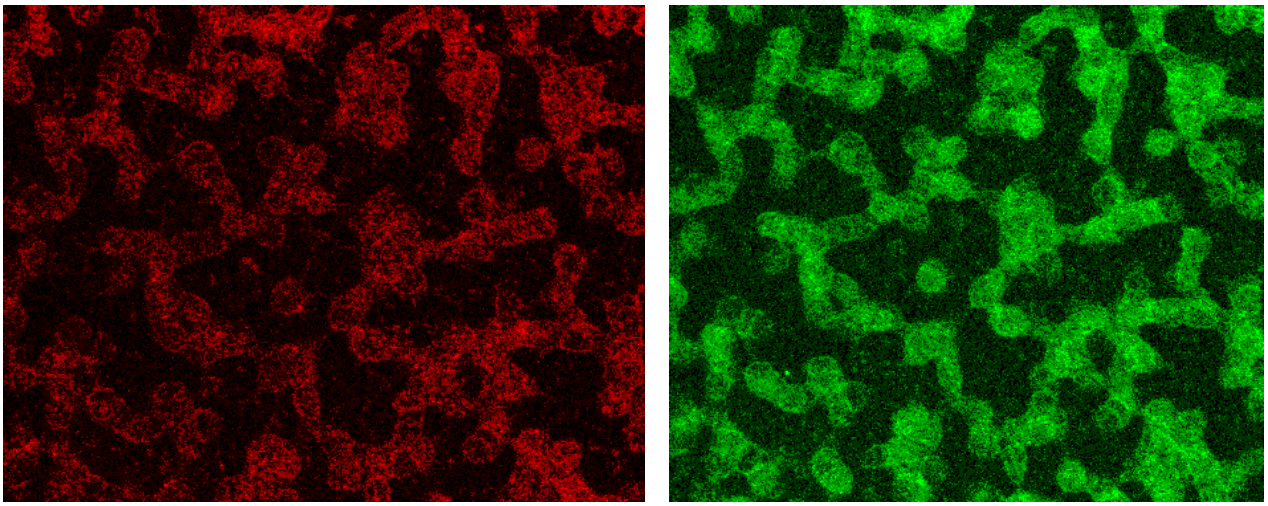

Figure 1: Double stained erythrocytes in autologous plasma (actin: left image in red; band4.9: right image in green; primary magnification 1:63; zoom factor: 2.1).

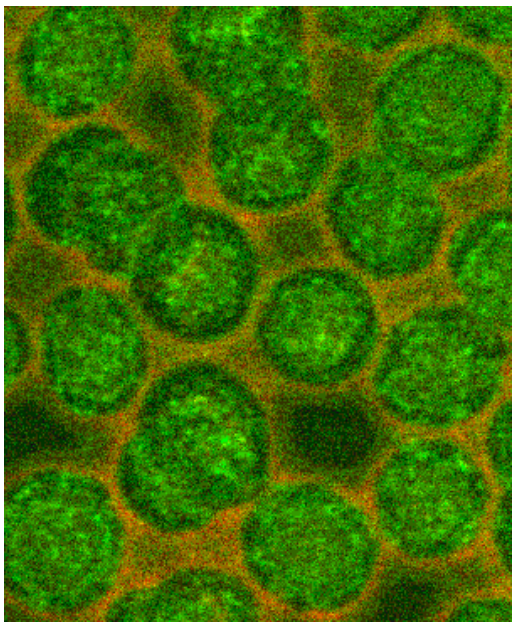

Figure 2: Band4.9-staining of erythrocytes in autologous plasma (primary magnification 1:63; zoom factor: 5).

Figure 3 shows a merger of the red-/green-channels of Figure 1. Co-localizations of actin and band4.9 - indicated by the yellow colour - were very rare.

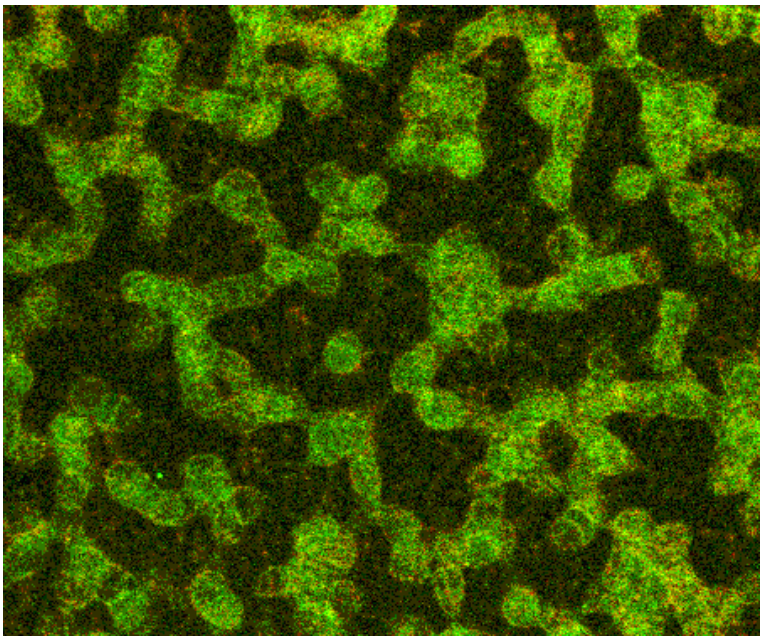

Figure 3: Merger of red (actin)- and green (band4.9)-channels of Figure 1 (Primary magnification 1:63; zoom factor: 2.8).

Figure 4 shows double stained erythrocytes after suspension in a plasma/Iodixanol - 320 mixture (Iodixanol-320 30% v/v) in the red (actin, left image) or the green (band4.9, right image) channel of the confocal laser scanning microscope.

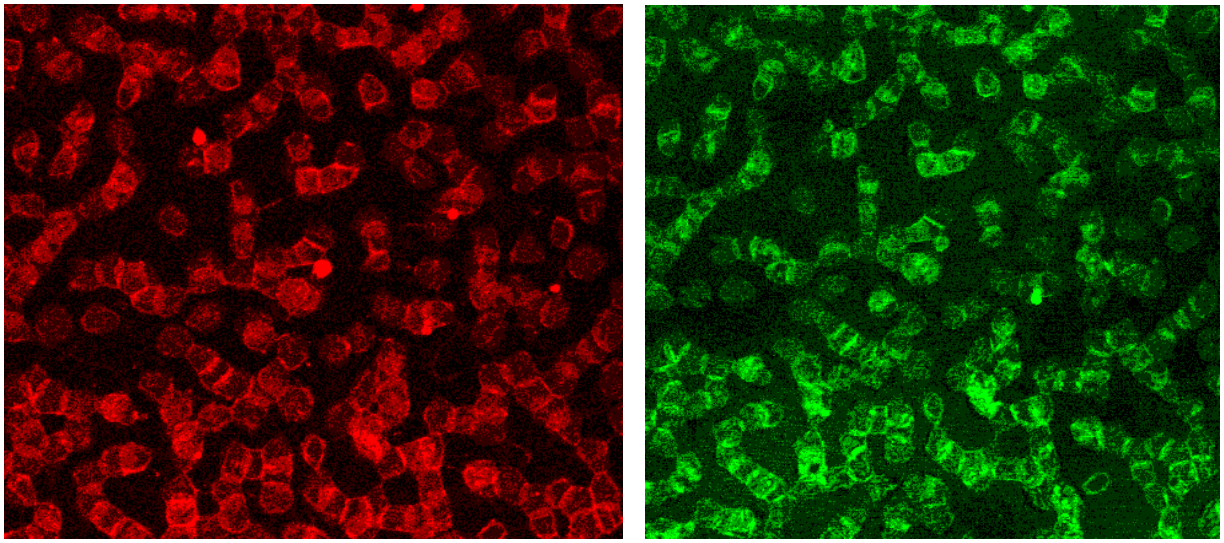

Figure 4: Actin (left) and band4.9 double-stained (right) erythrocytes after suspension in a Plasma/Iodixanol-320 mixture (Iodixanol-320 30% v/v) (Primary magnification 1:63; zoom factor: 2.1).

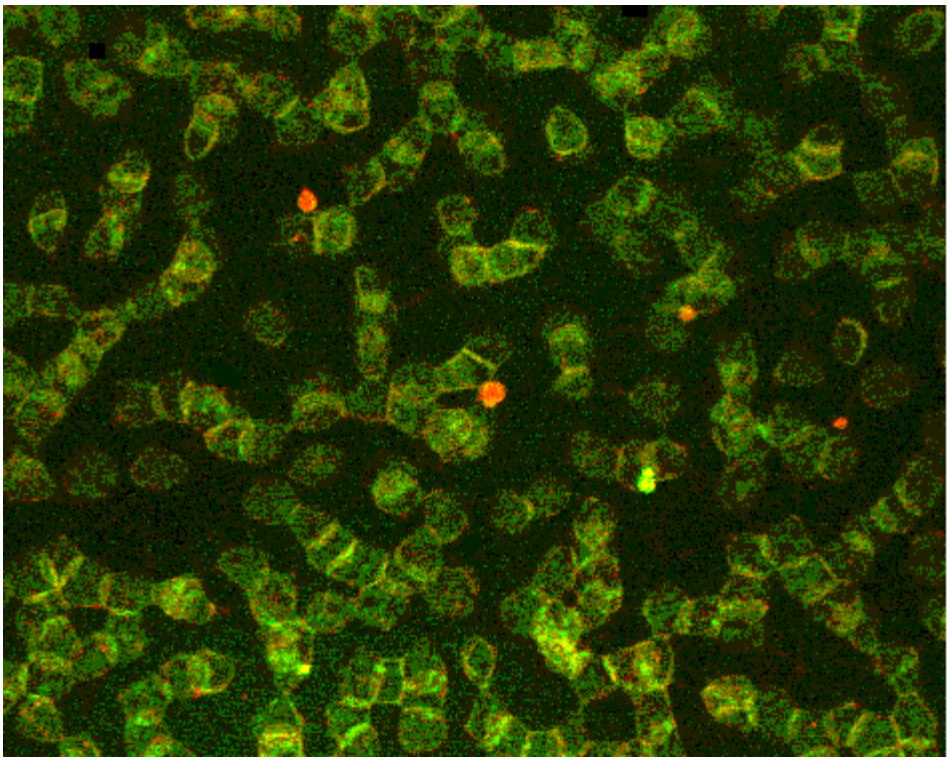

Figure 5: Merger of red (actin)- and green (band4.9)-channels of Figure 4 (Primary magnification 1:63; zoom factor: 2.8).

The distribution of band4.9 in the cells was often similar to cells suspended in autologous plasma. But in some cells thick knob-like aggregates of possibly polymerized actin bundles appeared. Whereas band4.9 in the cytoskeleton of most cells appeared to be in a round to spheroid shape, in some cells it was box-like arranged (see Fig. 5).

Figure 6 shows double stained erythrocytes after suspension in a plasma/Iopromide-370 mixture (Iopromide-370 30% v/v) in the red (actin, left image) or the green (band4.9, right image) channel of the confocal laser scanning microscope.

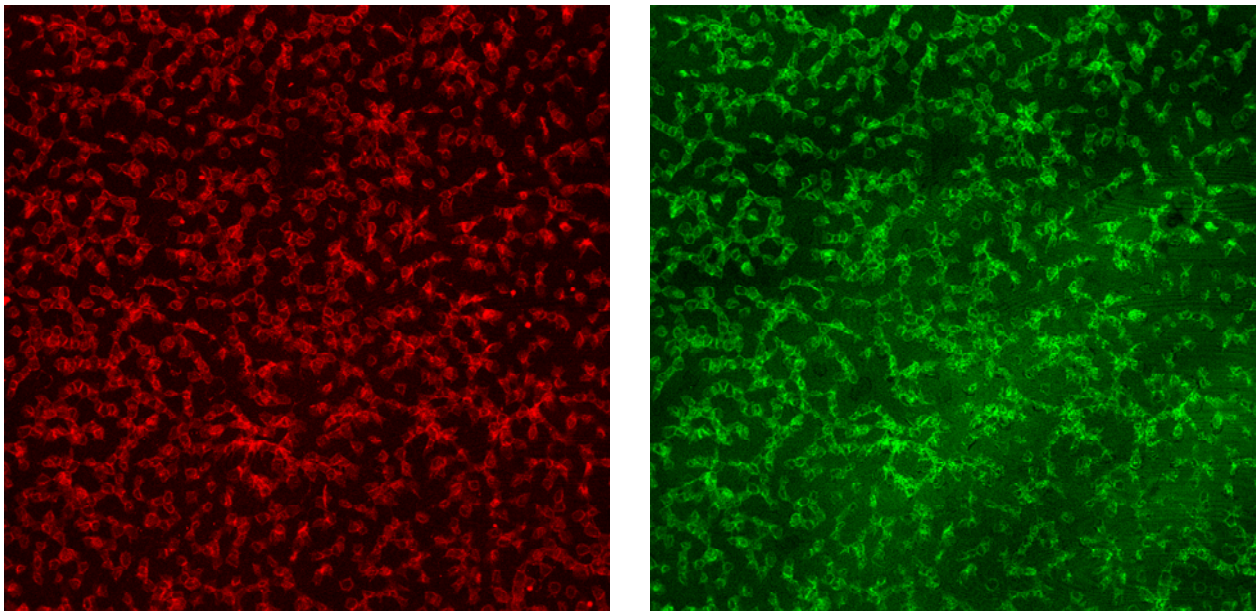

Figure 6: Actin (left) and band4.9 double-stained (right) erythrocytes after suspension in a Plasma/Iopromide-370 mixture (Iopromide-370 30% v/v). (Primary magnification 1:63; zoom factor: 1).

In practically none of the cells the rounded conformation of the membrane cytoskeleton - as it appeared in cells suspended in autologous plasma (see Fig. 2) - was found. Both RCM more or less induced a relocation of band4.9 from the centre of the erythrocytes to the cell periphery. While a great number of knob-like structures prevailed in central cell parts after addition of Iodixanol-320 (see Fig. 7, left), after Iopromide-370 band4.9 was concentrated as coarser knob-like structures at the cell periphery and in most cases the cell centers were devoid of band4.9 (see Fig. 7, right).

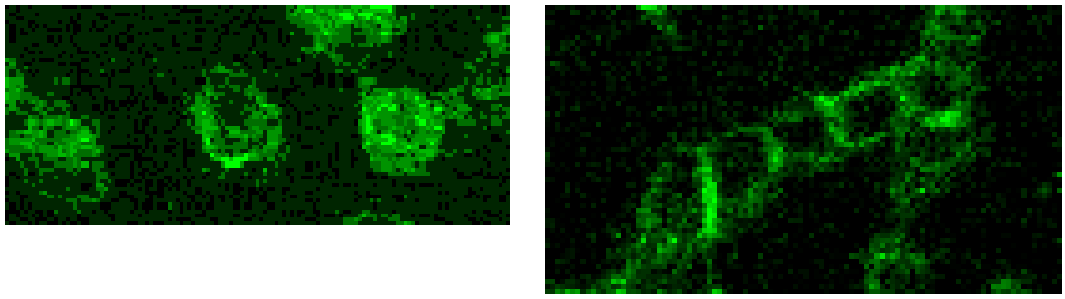

Figure 7: Band4.9 stained erythrocytes after suspension in a Iodixanol-320/plasma mixture (left) or in a Iopromide-370/plasma mixture (right) (Details of Fig. 4, right and Fig. 6, right).

The box-like arrangement of bands of band4.9 as well as the dissociation between colours red (actin) and green (band4.9) were more pronounced after suspension of erythrocytes in a plasma/Iopromide-370 mixture compared to the suspension of cells in a plasma/Iodixanol-320 or in autologous plasma (see Fig. 8).

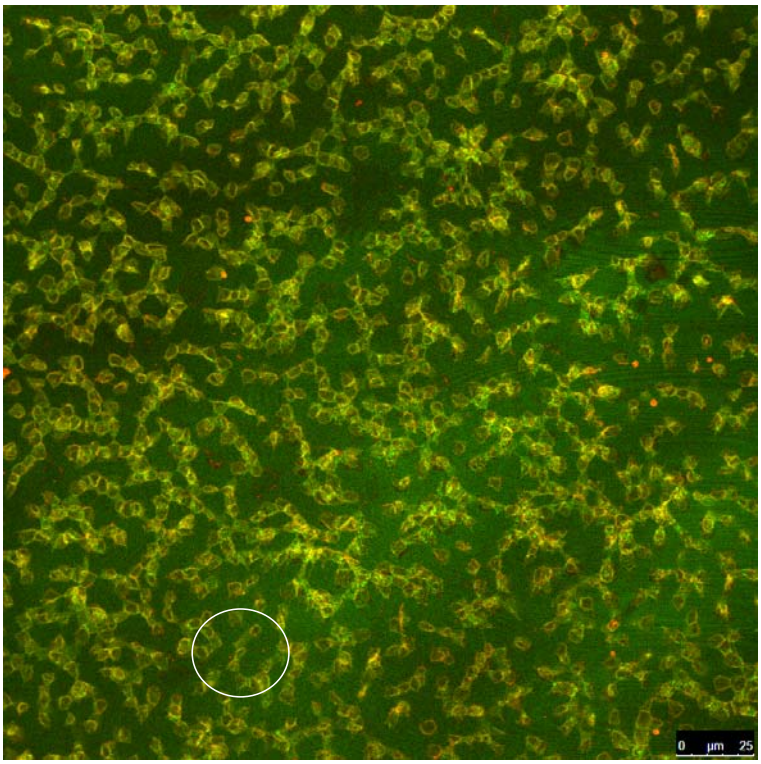

Figure 8: Merger of red (actin)- and green (band4.9)- channels of Figure 6 (Primary magnification 1:63; zoom factor: 1).
White circle shows an area with stained actin oligomers outside of erythrocytes, which is detailed in Fig. 12.

4. Discussion

Radiographic contrast media added to autologous plasma prior to suspension of erythrocytes, induced changes in the arrangement of band4.9 in human erythrocytes where the changes induced by Iopromide-370 (Fig. 8 and Fig. 9, right) were by far exceeding those induced by Iodixanol-320 (Fig. 5 and Fig. 9 middle). The knob-like and not quite homogeneous distribution of band4.9 in erythrocytes suspended in autologous plasma (see Fig. 2 and Fig. 9, left) - as was described by Lalle et al. for murine erythrocytes [31] - appeared in a similar manner in human erythrocytes suspended in a plasma/Iodixanol-320 mixture (see Fig. 5 and Fig. 9, middle), which

is not trivial because the membrane anchoring structure for band4.9 differs e.g. the GLUT1 receptor is missing in the membrane of adult mice [28].

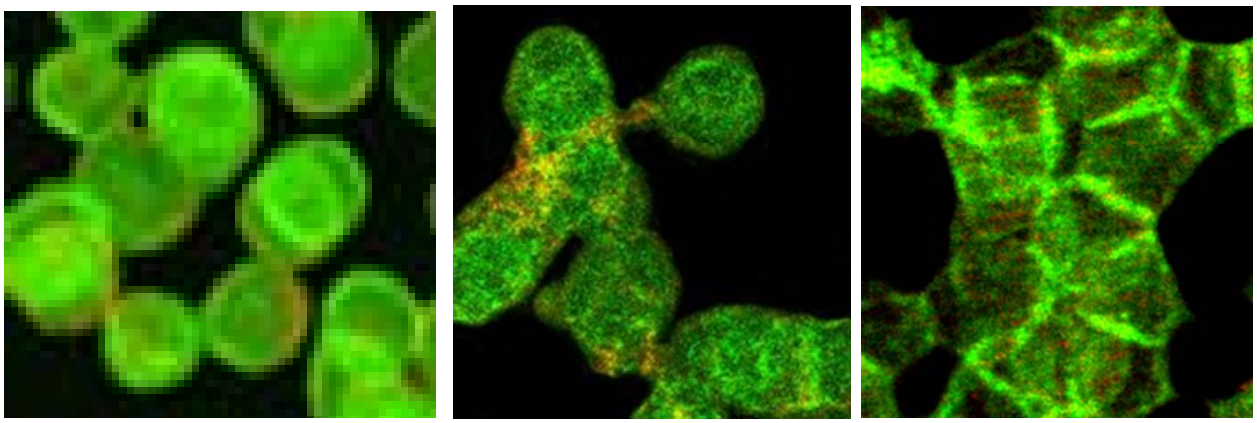

Figure 9: Details of the actin and band4.9 distribution in erythrocytes suspended in autologous plasma (left), in a plasma/Iodixanol-320 mixture (middle) or in a plasma/Iopromide-370 mixture (right).

There was a drastic change of band4.9 arrangement in erythrocytes after suspension in a plasma/Iopromide-370 mixture (Fig. 8 and Fig. 9, right). Band4.9 arrangement was strongly inhomogeneous now; it was arranged in broad bands and box-like along the rim of the aggregated erythrocytes and, not so pronounced, also in the few single cells.

Another major finding was the generation of small protrusions with varying diameters, more or less coated at least with actin, now dissociated from band4.9 after the incubation of the erythrocytes in a plasma RCM/mixture (see Fig. 5 and 8).

Cell protrusions with possibly polymerized and bundled actin appeared (see Fig. 11, left and right) which were not found in erythrocytes suspended in autologous plasma (in line with a former study of band4.9 influence on the stability of the murine erythrocyte membrane [29]).

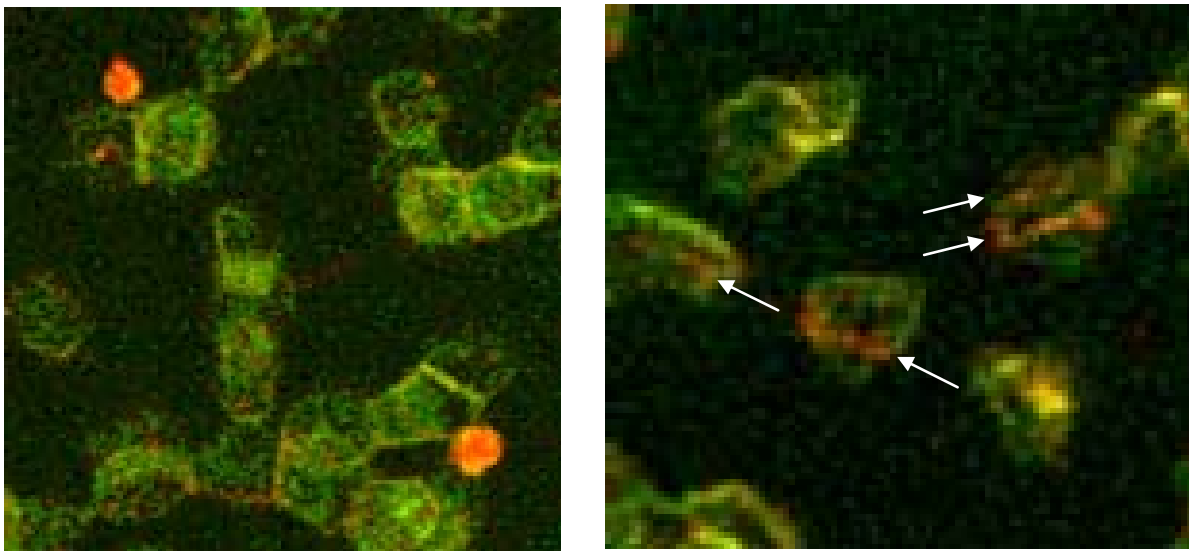

Figure 10: Erythrocytes suspended in a plasma/Iodixanol-320 mixture (left, magnified detail from Figure 5) or in a plasma/Iopromide-370 mixture (right, magnified detail from Figure 8; arrows mark transcellular long bands of actin).

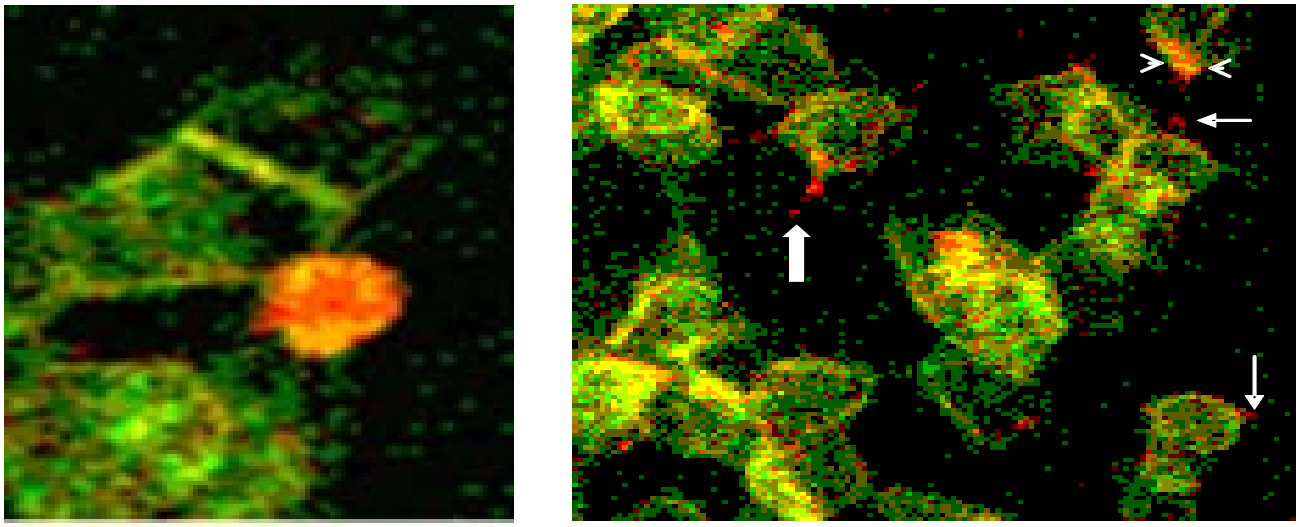

Figure 11: Protrusions at least coated with actin after adding Iodixanol-320 (left, magnified detail from Figure 5) or Iopromide-370 (right, magnified detail from Figure 8; arrowheads mark yellow bands associated with agglomerations of red colour probably indicating ring-like structures as known from exocytotic processes, white arrows point to aggregated actin still in conjunction with the cell membrane, broad arrow marks extracellular actin).

While in cells in autologous plasma nearly no protrusions with dissociated actin were visible, a few bigger protrusions with actin either dissociated or not dissociated from

band4.9 appeared after adding Iodixanol-320 to the plasma (Fig. 11, left and Fig. 10, left): See yellow stain around red stain in protrusions of Fig. 11 left, indicating the presence of actin and band4.9 and a very close neighbourhood of both, closer than before addition of Iodixanol-320. This could mean that most junctional complexes were intact with the exemption that some contacts between band4.9 and GLUT 1 were lost and band4.9 got considerably nearer to actin (co-localization with yellow shift of colours) hypothesized to be due to the loss of traction of the GLUT 1 receptor. A lot of small protrusions with dissociated actin were induced by Iopromide-370 (Fig. 8 and Fig. 11, right: Almost no yellow stain was seen in cell protrusions after adding Iopromide-370 [see Fig. 11, right; normal white and broad white arrows]) revealing that band4.9 was dissociated completely from actin. In some

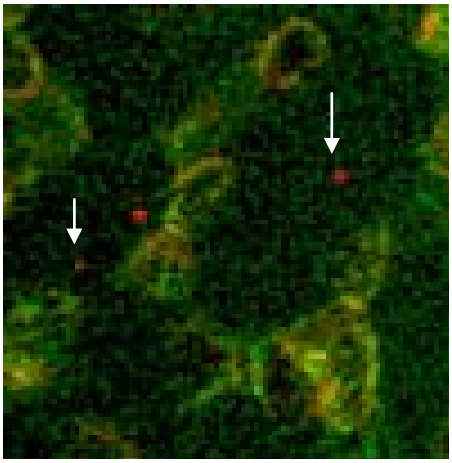

Figure 12: β-actin antibody stained actin oligomers outside of erythrocytes marked by white arrows (after adding Iopromide-370).
(Detail from Fig. 8 in white circle).

cases yellow bands were associated with agglomerations of red colour (see white arrow heads) probably indicating ring-like structures as known from exocytotic processes [42]. Considering the arrangement of band4.9 in bands probably in conjunction with spectrin, this could indicate both, the dissociation of the band4.9-/GLUT1-contact and/or the dissociation of the band4.9-/actin-contact while the band4.9 -/spectrin-contact was maintained. Smaller or greater amounts of sometimes

seemingly bundled actin and band4.9 in filaments were found apart from other band4.9 containing structures (see Fig. 10 right).

The dissociation of actin from other components of the erythrocyte membrane cytoskeleton – spectrin and band3 – had been observed in earlier studies already [18]. Now, this study revealed that Iodixanol-320 and Iopromide-370 induced different distributions of actin in the membrane cytoskeleton. Whereas dot-like red stain prevailed at the rim of erythrocytes suspended in autologous plasma and a similar distribution of the dot-like red stain was also in erythrocytes suspended in a plasma/Iodixanol-320 mixture, there was a different distribution of partly dot-like and partly fibrillar red stain (Figs. 9-11, right; see white arrows in Figs. 10 and 11 right) in erythrocytes suspended in a plasma Iopromide-370 mixture.
Not only was the arrangement of actin in the membrane skeleton of erythrocytes different after incubation in different plasma/RCM mixtures, actin oligomers outside erythrocytes were mainly found when cells were suspended in plasma/Iopromide-370 mixtures (see Fig. 12, white arrows). Beside extracellular actin oligomers, also band3 oligomers had been described outside erythrocytes in a former study [17].

This study revealed for the first time a clear loss of actin-oligomers after contact of erythrocytes with Iopromide-370, demonstrated by a sequestration of actin out of the tips of the spicules giving evidence that the echinocyte formation provoked by Iopromide-370 seems to be associated with an exocytotic-like process – a mechanism well known from somatic cells containing nuclei [1,6], but up to now not described in erythrocytes.
Figure 13 shows in image C a merger of Image A (optical microscopy, trans illumination) and image B (confocal laser scanning microscopy after immune staining of β-actin), both in transparency of 50%. Actin was clearly sequestrated from the spicules of echinocytes.

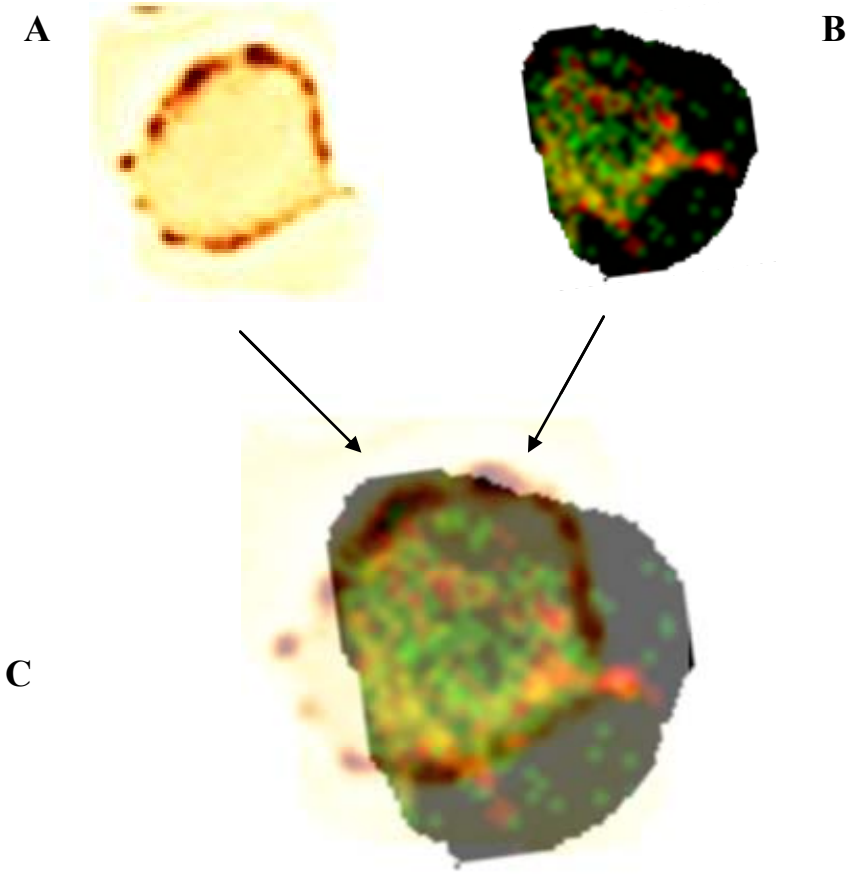

Figure 13: Merger (C) of two images A (optical microscopy) and B (confocal laser scanning microscopy, actin stained).

It might be hypothesized that this loss of actin critically reduced the number of binding elements, usually necessary to link membrane cytoskeleton and erythrocyte membrane in a satisfactory manner, so that neither deformation and red cell function would be compromised nor would a senescence antigen be brought forward, so that e.g. echinocytes could revert then to competent discocytes. A substantial reduction of actin could thus be a reason that echinocyte formation is no longer reversible: in fact was a certain fraction of echinocytes - after their resuspension in autologous plasma - found not to switch back to discocytes [41]. It might be hypothesized further that

older erythrocytes particularly – with less cell volume, decreases in haemoglobin [19] and cell function [44] – could be the prime targets in these cases.

Band4.9, earlier described as a trimeric assembly of two 48-kDa polypeptides and one 52 kDa polypeptide [3], more recently appeared as a monomeric polypeptide with actin binding motives in the well structured C-terminal headpiece domain and in the intrinsically disordered C-terminal domain thereby allowing the parallel bundling of actin. In human erythrocytes about 43.000 copies of band4.9 were found [24]. It was assumed that one band4.9 polypeptide is associated with one actin oligomer *in vivo* [29]. When the headpiece domain was not available the dimer-tetramer equilibrium of spectrin was not influenced assumed to be due to binding of spectrin to the non-ordered N-terminal core domain of band4.9 [9,29]. This hypothesis from Chishti´s group might be confirmed by our results. Since the small monomeric band4.9 in normal erythrocytes would not arrange in multimeric filaments but in knob-like structures, the observed box-like arrangement of band4.9 in dense "bands" (see Fig. 9, right) - after suspension in a plasma/Iopromide-370 mixture - is most probably indicating that these dense bands consisted of spectrin (as shown in a recent publication [17]) decorated by monomeric band4.9 still linked to the spectrin. This would afford a conserved direct link between band4.9 and spectrin even after interaction with RCM. However, some or more of the actin bound to the headpiece and the N-terminal domains of band4.9 was subjected to dissociation – recognizable by the separated actin bundles (not bundled by band4.9 since red coloured structures were without any accompanying green colour in Figs. 9 and 11, right) found directly beneath band4.9 "bands". This RCM-induced dissociation of the actin-band4.9-construct of the junctional complex might show influences of RCM on both, the headpiece and the N-terminal domains of band4.9. This pathophysiological process could be one mechanism for cell deterioration summarized as "chemotoxicity of radiographic contrast media" first described by Peter Dawson in 1985 [11] and which is still unclear until today.

It is also unclear whether RCM effected a dissociation of band4.9 from actin only or a dissociation also from the glucose transporter 1 (GLUT1), which was identified as a receptor for band4.9 in the erythrocyte membrane [28]. The formation of thick spectrin bands as already shown earlier [17], and now demonstrated to coincide with bands of band4.9 accumulating at the rim of the cells are thought to be a consequence of a partial dissociation of the cytoskeleton from the band4.9-depending membrane anchors (shown to be not so weak in principle because they could resist biochemical dissociation upon detergent extraction [28].

Whether adducin – another component of the junctional complex – and also binding actin to the GLUT1 receptor was dissociated, too, from its anchor needs to be analyzed in the next future. This is thought to be less probable because then a very high percentage of anchors binding the erythrocyte skeleton to the membrane would no longer be there rendering the erythrocyte membrane extremely fragile.

5. Conclusion

Iopromide-370 induced a completely different distribution of band4.9 of the junctional complex stained by FITC coupled and affinity-purified monoclonal antibodies while Iodixanol-320 did not modify the distribution of band4.9 as compared to erythrocytes suspended in autologous plasma. Similar results were found with respect to the distribution of actin. In addition, also a process resembling exocytosis of particles at least coated with actin could be demonstrated after incubation of erythrocytes in a Iopromide-370/plasma mixture.

This study revealed for the first time RCM-dependent differences in band4.9 engagement, a possible pathophysiological mechanism for the chemotoxicity of radiographic contrast media described by Dawson [11].

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