Tetraspanin CD9 is involved in the migration of retinal microvascular endothelial cells

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Abstract. Members of the tetraspanin protein family are modulators of several fundamental cellular processes in various cell types. However, expression and function of these proteins have not been studied in microvascular endothelial cells despite their (patho-)physiological importance. Western blotting, FACS or RT-PCR analyses confirmed that CD9 and other tetraspanins are expressed in immortalized microvascular endothelial cells of the bovine retina (iBREC). In subconfluent cultures, most of the detected CD9 was located intracellularly as well as in the plasma membrane at cell-cell contact sites and in long spike-like extensions, whereas cells in confluent cultures predominantly showed plasma membrane staining. In wound healing assays, CD9 delocalized from the plasma membrane to its intracellular compartment in cells located at the gap border, and the gap closure was retarded by the addition of an anti-CD9 antibody. Migration of iBREC towards fibronectin and their adhesion to fibronectin were also strongly inhibited in the presence of an anti-CD9 antibody whereas other anti-tetraspanin antibodies had no effect. In summary, iBREC express members of the tetraspanin family of which CD9 was demonstrated to have a function in migration and adhesion of these cells.

Introduction

Tetraspanins are transmembrane proteins consisting of two extracellular loops and short intracellular ends that are linked through four transmembrane domains. Members of this protein family are implicated in various cell types in the regulation of differentiation and fundamental processes including proliferation, adhesion and motility (1,2). Tetraspanins can modulate cellular functions by organizing specialized 'tetraspanin-enriched' membrane microdomains, in which they form complexes with other tetraspanins and different types of other membrane proteins, thereby providing an important structural component of cellular signaling. It has been demonstrated that many tetraspanins can bind to integrins and related proteins to form functional complexes involved in cell-cell and cell-extracellular matrix (ECM) interactions (3,4). Accordingly, they are crucially involved in important physiological processes, e.g CD9 in sperm-egg fusion in mammals (5), and pathogenic mechanisms. In many types of malignant diseases, e.g. breast and cervical cancer (6,7) and retinoblastoma (8), the level of expression of CD9 was found to correlate, in most cases inversely, with the stage of tumor progression, appearance of metastases or poor clinical outcome. CD9 has also been described to promote infection of mammary carcinoma cells by adeno-associated virus type 2 (9) which is interesting in view of novel therapeutic approaches including intraocular gene transfer with this vector (10). CD9, CD81 and CD82 are involved in the late sustained response to an injury in the rat retina (11), and endothelial cell-expressed CD151 is important for pathological vascularization but not normal angiogenesis during embryonic development, as recently observed in CD151-deficient mice (12). In endothelial cells of the macrovasculature, the roles of the tetraspanins CD9, CD81, CD151 and CD63 in adhesion, wound healing and migration were shown (13-15). In contrast, tetraspanin expression and their functions in microvascular endothelial cells of the retina (REC) have not been studied yet despite their (patho-) physiological importance; cellular processes that are deregulated in endothelial cells in retinal diseases such as diabetic retinopathy are the same for which tetraspanins are known modulators, especially migration and adhesion to components of the ECM and, therefore, a contribution of these proteins to the genesis of such diseases can be assumed. We show here that the tetraspanins CD9, CD63 and CD151 are expressed in distinct compartments in immortalized REC of the bovine retina (iBREC) and that CD9 is involved in migration and adhesion of iBREC, of which inhibition of wound healing by an anti-CD9 antibody in an *in vitro* assay was indicative.

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Materials and methods

Antibodies, reagents and media. Mouse monoclonal antibodies (mAbs) binding to bovine CD9 (clone IVA50), bovine CD63 (clone CC25), or human CD81 (clones ID6 and

B399), and rabbit polyclonal antibodies specific for human vascular endothelial cadherin (VECad) (16) were obtained from Acris (Hiddenhausen, Gemany); mAbs against human CD151 (clone 11G5a) and human CD9 (clone MM2/57) were from AbD-serotec (Dusseldorf, Germany). A mAb (clone 210127) and a rabbit polyclonal serum specific for human CD151 were from R&D Systems (Wiesbaden, Germany) and Protein Tech Group (Chicago, IL, USA), respectively. Rabbit polyclonal antibodies binding to human von Willebrand factor (vWF) and an mAb directed against human α-smooth muscle actin (αSMA; clone 1A4) were purchased from Dako Cytomation (Hamburg, Germany). Mouse mAb against Golgin 97 (clone CDFX), a protein located in the Golgi apparatus (17), was purchased from Biozol (Eching, Germany). AlexaFluor® 594- or AlexaFluor® 488-conjugated detection antibodies (IgG H+L chains) were from Invitrogen (Karlsruhe, Germany) and FITC-labeled secondary antibodies [$F(ab')_2$] were from Dianova (Hamburg, Germany). Horseradish peroxidase (HRP)-conjugated anti-mouse antibodies and the SuperSignal® West Dura Extended Duration Substrate kit were obtained from Pierce (Bonn, Germany) as well as precast SDS polyacrylamide gels and StartingBlock™ (TBS) blocking buffer. The Complete™ premixed EDTA-free protease inhibitor cocktail was from Roche Diagnostics (Mannheim, Germany). Polyvinylidene difluoride (PVDF) membranes were purchased from Pall Europe (Dreieich, Germany). RNeasy® kits for isolation of RNA as well as primer/probe sets and the QuantiTect® Multiplex PCR kit for quantitative RT-PCR were obtained from Qiagen (Hilden, Germany). Reverse transcription was performed with the MultiScribe enzyme from Applied Biosystems (Darmstadt, Germany). Synthetic oligonucleotides used as PCR primers were prepared by Thermo Electron (Ulm, Germany). For agarose gel electrophoresis, E-gels (2%) from Invitrogen and the peqGold 100 bp DNA-Ladder plus (Peqlab, Erlangen, Germany) were used. Complete microvascular endothelial growth medium (ECGM-nG) based on MCDB131 (18) was from Promocell (Heidelberg, Germany). ECGM-nG was supplemented with premixed additives resulting in final concentrations of 0.4% ECGS/H, 5% fetal calf serum, 10 ng/ml epidermal growth factor, 1 g/l glucose, 10 mM $MgCl_2$ and 2.8 μM hydrocortisol. To obtain ECGM-hG, additional glucose was added to a concentration of 4.5 g/l. Basal medium (EBGM, Promocell) was supplemented only with 2.8 μM hydrocortisol.

Cell cultivation. Immortalized microvascular endothelial cells from bovine retina (iBREC) were established by transfection of primary retinal endothelial cells with plasmid pCI-neo hTERT encoding the catalytic subunit of human telomerase reverse transcriptase under control of a CMV promotor/enhancer (19). This homogenous cell line expresses endothelial marker proteins vWF and VECad but not αSMA, a marker for vascular smooth muscle cells (19,20). iBREC proliferate and can be stimulated by growth factors such as parental primary BREC (19). Cells were maintained in ECGM-nG in 25-cm^2 flasks coated with human fibronectin (50 μg/ml; BD Biosciences, Heidelberg, Germany) in a humidified atmosphere containing 5% CO_2 at 37˚C and were passaged every 3 days. To ensure expression of hTERT during cultivation of iBREC, all media were supplemented with 300 μg/ml Geneticin (Invitrogen). The effects of the altered experimental conditions were studied in high-glucose medium (ECGM-hG; 4.5 g/l glucose) and on laminin-coated (10 μg/ml, BD Biosciences) cell culture flasks. iBREC were always detached with accutase (PAA, Coelbe, Germany). Secondary cultures of HUVEC (Promocell) were cultivated on fibronectin culture flasks according to the supplier's protocol.

Immunofluorescence staining. iBREC cultivated on two-chamber slides (BD Biosciences or Nunc, Wiesbaden, Germany) coated with fibronectin (or laminin) were fixed in methanol/acetone or methanol at -20˚C, followed by an optional incubation in 0.25% NP-40 (Roche) in PBS at 4˚C for 5 min and blocking with 10% donkey serum (Dianova)/PBS for 30 min at room temperature. Slides were then incubated for 1 h at room temperature with primary antibodies specific for CD9 (clones IVA50 and MM2/57; 1 μg/ml), CD81 (clones ID6 and B399; 2.5-10 μg/ml), CD63 (clone CC25, 1:40-1:80), CD151 (clones 11G5a and 210107; 2.5-10 μg/ml) or VECad (6 μg/ml), respectively, diluted in 1% donkey serum in PBSd. A prediluted mAb against αSMA was used as negative control. After washing with PBS, the secondary antibody of the required specificity (AlexaFluor 594, 0.5 μg/ml or AlexaFluor 488, 1 μg/ml in 1% donkey serum/PBS) was added for 1 h at room temperature. For double staining, appropriate FITC-conjugated $F(ab')_2$ fragments were used (5 μg/ml in 1% donkey serum/PBS) to detect the bound antibody, and the binding of the second antibody was visualized with an AlexaFluor 594 conjugate. Eventually, chamber slides were washed, and the cells were embedded in VectaShield or VectaShield-DAPI (Vector Laboratories, Axxora, Grünberg, Germany), before they were examined by fluorescence microscopy (Leica DM4000B, Bensheim, Germany). Identical staining patterns observed with different detection antibodies were considered indicative of the specificity of the used primary antibodies.

Flow cytometry. iBREC of confluent or semi-confluent cultures were washed three times with cold PBSd, scraped off the flasks, collected by centrifugation and re-suspended in PBSd containing 1% donkey serum. Cells ($4x10^6$) were incubated in 250 μl with the tetraspanin-specific antibody (10 μg/ml αCD9, 20 μg/ml αCD151 or αCD63 diluted 1:40) or without the primary antibody (control) for 1 h on ice. After removal of the non-bound primary antibodies, cells were washed with PBS and incubated with the AlexaFluor 488-conjugated secondary antibody (4 μg/ml in 250 μl) for 45 min on ice. After washing the cells three times with cold PBSd, cells were re-suspended in 500 μl PBSd and 0.002% EDTA. Propidium iodide (4.5 μg/ml) was added to exclude membrane-permeable cells from the determination of antigen-specific fluorescence for which a FACSCalibur (BD Biosciences) with CellQuestPro 2.0 software was used.

Western blot analyses. To prepare whole cell extracts, $2x10^6$ cells were detached by scraping, re-suspended in 300 μl cold lysis buffer (40 mM Tris-Cl, 150 mM NaCl, 1% Brij 97, pH 7.4, supplemented with the EDTA-free protease inhibitor

cocktail) and incubated on ice for 1 h. After centrifugation for 15 min at 10000 x g at 4˚C, proteins in the supernatant were separated by electrophoresis in a 12% SDS polyacrylamide gel under non-reducing conditions and transferred to a PVDF membrane by semi-dry electroblotting. The membrane was blocked with StartingBlock™ (TBS) blocking buffer for 1 h at room temperature and incubated with the primary antibody (1 μg/ml anti-CD9 or other anti-tetraspanin antibodies) overnight at 4˚C. Binding of these antibodies was detected with anti-mouse IgG HRP-peroxidase conjugates (0.1 ng/ml) and the SuperSignal® Substrate kit according to the supplier's protocol.

RT-PCR. RNA from confluent and non-confluent iBREC was extracted with an RNeasy kit, and cDNA was synthesized by reverse transcription with random hexamer primers. Primer sequences to amplify parts of the cDNA of bovine CD9, CD63, CD81, CD82 and CD151 were: CD9-F1: 5'-CGGTCAAAG GAGGCACCAAGTG-3'; CD9-F2, 5'-TGGACTATGGCTC CGATTCGACTC-3' CD9-R1, 5'-TCTGGACTTCCTTGAT CACCTCCTC-3'; CD63-F1, 5'-TGGCGGTGGAAGGAG GAATG-3'; CD63-R1, 5'-GGATCGATGCCGTCTGGTT GTC-3'; CD81-F1, 5'-CCTGCTCTTCGTCTTCAATT TCGTC-3'; CD81-F2, 5'-GCAGACCACCAACCTCCTGTA TCTG-3'; CD81-R1, 5'-GGCAAAGAGGATCACCAGG CAG-3'; CD82-F1, 5'-GGGTCAGCCTGCATCAAAGT CAC-3'; CD82-F2, 5'-TGGCGGCCTACGTCTTCATCAG-3'; CD82-R1, 5'-CACTTCACCTGAGCCTGCACGTAG-3'; CD151-F1, 5'-TCGACAACATGTGGCACCGTC-3'; and CD151-R1, 5'-CAGGTTCCTCCGCTCCTTGAAG-3'. PCR was performed with FastStart-Taq DNA-polymerase (Roche) at 94˚C for 2 min; 35 cycles at 94˚C for 30 sec, 60˚C for 45 sec and 72˚C for 1 min; and a final polymerisation step at 72˚C for 7 min.

For quantitative RT-PCR, an ABI PRISM 7700 Gene Analyzer (Applied Biosystems) was used in combination with a QuantiTect® Multiplex PCR kit (Qiagen). The PCR conditions were: 95˚C for 15 min; 45 cycles at 94˚C for 45 sec, 56˚C for 45 sec, and 76˚C for 45 sec. Primer/probe sets specific for bovine CD9, CD63, CD151 and ß2-microglobulin (B2M) were synthesized by Qiagen: CD9-forward, 5'-CGAGGAC ACCTACAACAA-3'; CD9-probe, 5'-GAAGAACAAGGAC GAG-3'; CD9-reverse, 5'-CGGTCAAACCACAGCAGT-3'; CD151-forward, 5'-GTACCTGCTCTTCACCTT-3'; CD151-probe, 5'-TCTGGCTGGCTGGTCT-3'; CD151-reverse, 5'-GG CTGATGTAGTCGCTCTT-3'; CD63-forward, 5'-GGAGA ACTACTGTCTTATGAT-3'; CD63-probe, 5'-CCATCTT CTTGTCCCT-3'; CD63-reverse, 5'-CTCTAAACACAT AGCCAGCAA-3'; B2M-forward, 5'-GTCCTCCAAAGA TTCAAGTGTA-3'; B2M-probe, 5'-AAGACACCCACCA GAA-3'; and B2M-reverse, 5'-TGGGACAGCAGGTAGA AAGA-3'. The minor groove-binding probes were labelled either with FAM (CD9-, CD151- or CD63-probe) or with Yakima Yellow (B2M-probe). Recorded fluorescence signals were analysed with Sequence Detector 1.7 software, and the relative expression of the analysed tetraspanins was calculated with the $\Delta\Delta C_t$ method (21).

Wound healing assays. Wound healing/migration assays were performed essentially as previously described (13,22). iBREC were grown to confluency on fibronectin-coated two-chamber slides in ECGM-nG and washed twice with PBSd. The cell monolayer was disrupted with a cell scraper (2 mm), and repair of the lesion was studied in ECGM-nG supplemented with 5 μg/ml fibronectin over 4 to 7 days. Localization of tetraspanins during lesion repair was monitored by immunofluorescence staining at different time points. Proliferation rates of cells located at the lesion margin or in the monolayer were measured with an *In situ*-cell proliferation kit-FLUOS (Roche) by quantification of their BrdU incorporation; cells were incubated with 10 μM BrdU for 2 h at 37˚C before fixation in freshly prepared 15 mM glycine, pH 2.0/70% ethanol at room temperature for 45 min. BrdU incorporation was detected after denaturation of the DNA (94 M HCl, 20 min at room temperature) using a fluorescein-conjugated mAb [clone BMG 6H8 $F(ab')_2$] according to the manufacturer's instructions. Stained nuclei and the total number of cells were counted in duplicate wells in six randomly chosen microscopic fields. Lesion repair was also investigated with this method in the presence of 7.5 μg/well (25 μg/ml) of monoclonal antibodies against CD9 or CD151 in comparison with an isotype-matched control antibody. In all experiments, the relative gap sizes at different time points during closure of the set lesions were estimated in quadruplicate wells in four randomly chosen microscopic fields.

Cell migration assay. Transmembrane cell migration assays were performed in a modified Boyden chamber (23) consisting of 12-well cell culture plates and inserts with a porous polyethylenterephthalate membrane (pore size 8.0 μm, Ø 1 cm^2; BD Biosciences). The lower compartment of a 12-multiwell plate was filled with serum-free medium (EBGM-nG or EBGM-hG) containing 5 μg/ml fibronectin (or 5 μg/ml BSA in control experiments), and inserted membranes were initially incubated for 1 h at 37˚C before 400 μl of serum-free medium was added to the insert. Anti-tetraspanin antibodies or an appropriate control antibody (25 μg/ml) were incubated with $4x10^5$ iBREC for 30 min at 37˚C in 400 μl serum-free medium. Of this cell suspension, 100 μl containing 10^5 cells was then placed on the preincubated membrane and migration of cells through the membrane pores was assessed after 20 h at 37˚C. Cells still on top of the membrane were scraped off, and cells which had migrated to the lower surface of the membrane were fixed in methanol at -20˚C for 15 min. Nuclei of migrated cells were stained by mounting the membrane in VectaShield-DAPI and visualized by fluorescence microscopy. Cells were counted in triplicate wells in 4 randomly chosen microscopic fields. Similar experiments were performed with iBREC that had been precultivated in ECGM containing 4.5 g/l glucose for several weeks. iBREC migration and its inhibition by an anti-CD9 antibody were also evaluated in serum-containing medium.

WST-1 cell adhesion and proliferation assays. iBREC ($5x10^3$) were cultivated in ECGM-nG and preincubated with 1.5 μg antibodies (15 μg/ml) against CD9, CD63, CD151 or a control antibody, respectively, for 30 min at 37˚C before cells were seeded in a fibronectin-coated well of a 96-well plate. After incubation for 1 h, attached cells were washed,

and the cell-mediated enzymatic conversion of WST-1 (Roche) was measured according to the manufacturer's protocol in 8 replicate wells.

To measure proliferation, iBREC were seeded at a density of 10^4/well in a fibronectin-coated 96-well plate and allowed to adhere in ECGM-nG or ECGM-hG overnight. Enzymatic conversion of WST-1, indicative of proliferating cells was recorded. The potential influence of anti-tetraspanin antibodies (addition of 0.5 μg mAb/well) on iBREC proliferation was investigated when cells had been allowed to adhere for 3 h. After incubation for 20 h, proliferating cells were quantified on the basis of WST-1 conversion.

Statistical analysis. The Mann-Whitney U test was used to analyze experimental data, and resulting p-values <0.05 were considered indicative of significant differences.

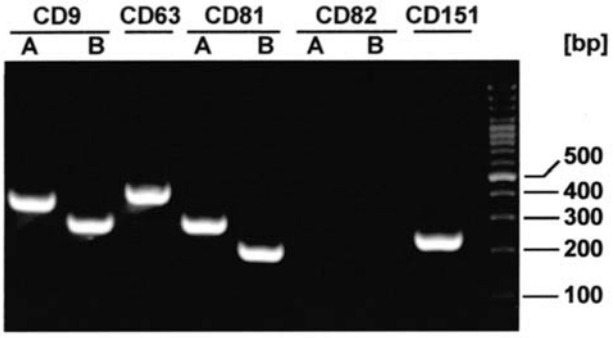

Figure 1. Expression of tetraspanin mRNAs in iBREC. cDNA was prepared from mRNA isolated from confluent cultures and amplified by PCR using different primer combinations. Specific PCR products, all of the expected sizes, confirmed expression of mRNAs encoding CD9, CD63, CD81, and CD151.

Results

Expression of tetraspanins CD9, CD63, CD81 and CD151 in iBREC. Expression of the tetraspanins CD9, CD63, CD81, CD82 and CD151 in immortalized microvascular cells isolated from the bovine retina (iBREC) (19) grown on fibronectin-coated surfaces was analyzed by RT-PCR, flow cytometry, Western blotting and immunofluorescence staining with specific antibodies. RT-PCR showed that mRNAs encoding CD9, CD63, CD81 and CD151 were present in iBREC, whereas CD82-specific amplification was not observed (Fig. 1). As measured by quantitative real time-PCR, the amounts of mRNAs specific for CD9 and CD151 were similar to that encoding ß2-microglobulin (on which normalization was based on), whereas expression of CD63 mRNA was significantly lower in the range of 1% of these quantities. The level of CD151 mRNA significantly ($p<0.05$) decreased to 1/3 of the initial value when the cell layer reached confluency. This notable effect was not observed for CD9 and CD63. In accordance with RT-PCR results, FACS analyses confirmed strong cell surface expression of CD9 in both non-confluent and confluent iBREC. The subcellular localization of CD9 was determined by immunofluorescence staining with anti-CD9 mAb of clone IVA50 (Fig. 2) which was superior to clone MM2/57 in this application. In non-confluent iBREC, an intracellular CD9-specific staining was seen as well as a pronounced perinuclear localization mainly concerning the Golgi apparatus which was confirmed by double-immunofluorescence with an anti-Golgin 97 antibody. The grid-like pattern of the intracellular staining also extended to the spike-like extensions of iBREC which seemed to mediate cell-cell contacts. In addition, contact sites of the plasma membranes of adjacent cells were usually CD9-positive. In confluent iBREC, CD9 staining was predominantly localized in the plasma membrane, but part of the protein was still detected intracellularly. In Western blot analyses, iBREC-expressed CD9 appeared, like in other cell types, as a characteristic double-band around 25 kDa under non-reducing conditions confirming the specificity of the antibody. Strong CD63-specific immunoreactivity was detected in intracellular vesicles in confluent and non-confluent iBREC (Fig. 2). A very small fraction of the total amount of CD63 co-localized with vWF indicating its presence in Weibel-Palade bodies, but most of the protein was, in accordance with the distribution in human umbilical vein endothelial cells (HUVEC) (14), detected in endosomes. The staining patterns of mAbs against CD9 and CD63 indicated that these proteins localized partly to similar compartments (Fig. 2). Available antibodies specific for human CD81 and human CD151 showed no cross-reactivity with the bovine tetraspanins when used for immunofluorescence staining, FACS analyses and Western blotting. Only mAbs 11G5a and 210127 against human CD151 gave rise to a diffuse intracellular staining in non-confluent iBREC, most likely associated with vesicles, which was hardly detectable in confluent iBREC (Fig. 3). Compared to iBREC cultivated on fibronectin-coated surfaces, this staining of vesicular structures was more intense in iBREC cultivated on laminin (Fig. 3). In some cells, stained spots were observed in the plasma membrane when cultivation on laminin was extended. However, HUVEC cultivated on fibronectin-coated surfaces showed the characteristic plasma membrane staining with mAb 11G5a (data not shown). The overall staining patterns were very similar with antibodies specific for CD151 and CD63 suggesting that these tetraspanins are mainly transported to the same intracellular vesicles in iBREC. Tetraspanin-specific immunostaining was not affected by cultivation of iBREC for several weeks in medium containing more glucose.

Relocation of CD9 and CD151 during lesion repair. A lesion was set to a confluent monolayer of iBREC cultivated in medium containing 5 μg/ml fibronectin by scraping off cells. During closure of the resulting 2- to 5-mm gap, the proliferation rate of the remaining cells and their expression of tetraspanins (CD9, CD63 and CD151) were studied by BrdU incorporation and immunofluorescence staining, respectively (Figs. 4 and 5). As early as 2-4 h after setting the lesion, cells started to form thin and long as well as short and broad extensions into the gap, and cells engaged in this process appeared to be larger than those within the monolayer. After one day, cells at the lesion margin began to lose contact with the monolayer (Fig. 4A) and started to migrate into the gap which was completely closed after 3-6 days depending on the initial size of the lesion. Cells at the lesion border but not in

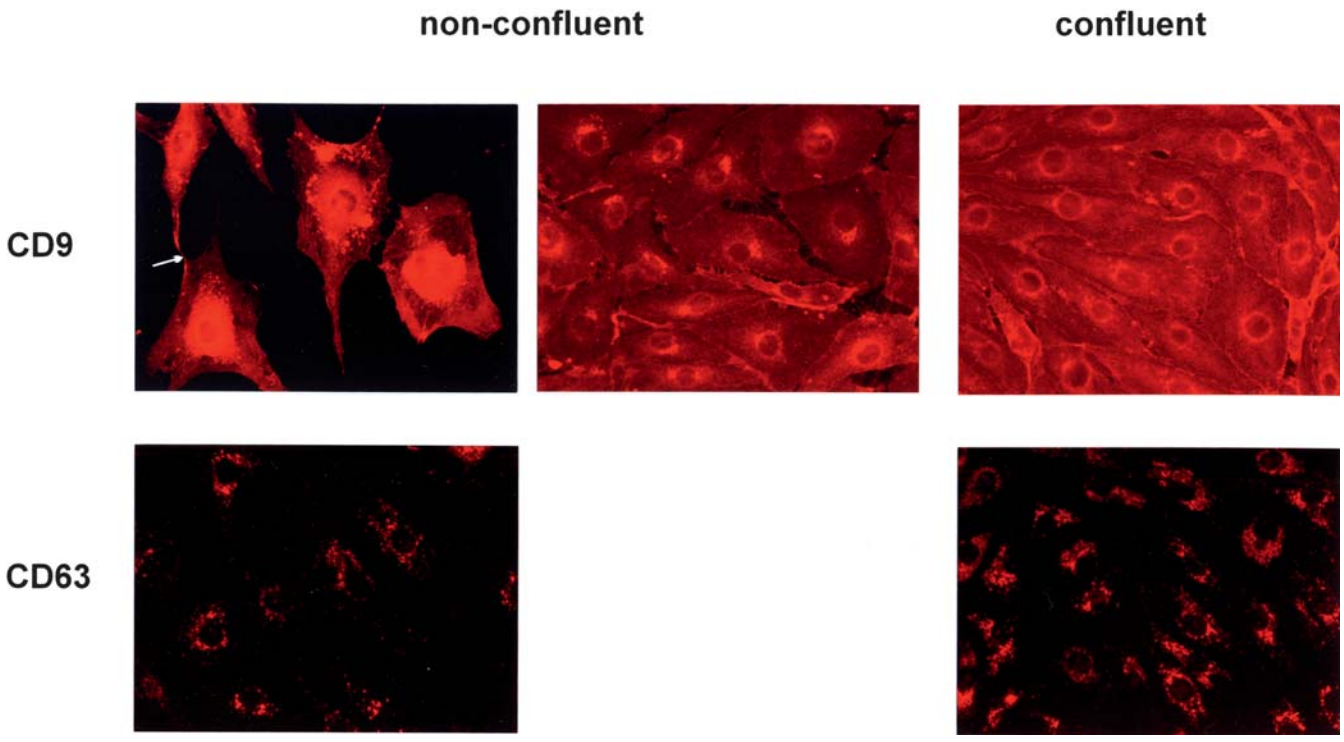

Figure 2. Immunofluorescence staining of iBREC with mAbs against bovine CD9 and CD63 in non-confluent and confluent cultures. Most of the detected CD9 was localized intracellularly in addition to a strong plasma membrane staining at cell-cell contact sites. Arrow marks thin spike-like extensions formed by iBREC which show strong CD9-specific immunoreactivity. CD63 staining was mainly found in intracellular vesicles.

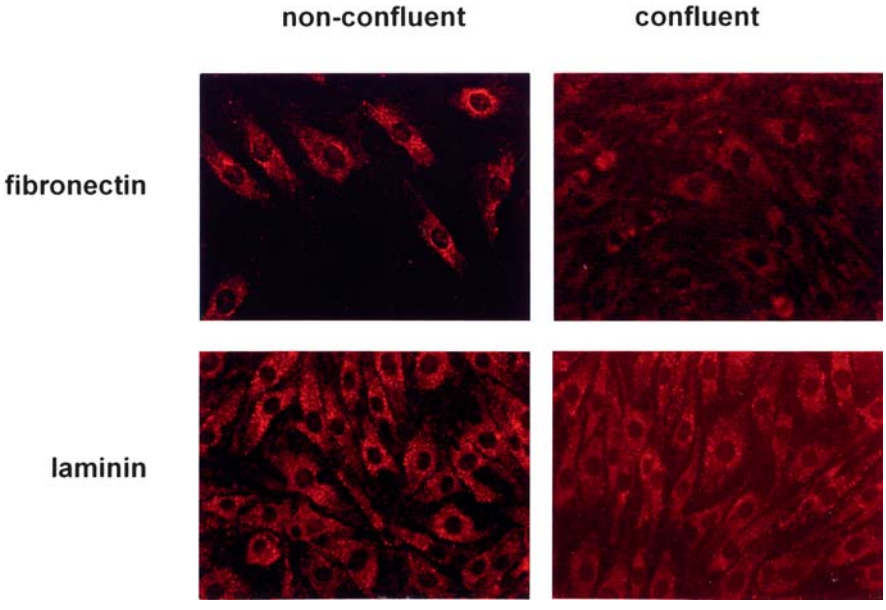

Figure 3. Immunofluorescence crossreactivity of non-confluent and confluent cultures of iBREC grown on fibronectin or laminin with mAb 11G5a directed against human CD151. Immunoreactivity was mainly associated with vesicles in non-confluent cultures, whereas it is much weaker in confluent cultures.

adjacent regions of the monolayer increased their proliferation rate within the first 24 h of *in vitro* wound healing, as measured by BrdU incorporation (Fig. 4B). As expected, elevated proliferation of involved cells was readjusted to normal values immediately after gap closure. Within the first 24 h after setting the lesion, the cellular distribution of CD9 in cells at the lesion margin began to change resulting in a pronounced staining of the protruding thin and long extensions (Fig. 5). No CD9-specific immunoreactivity was seen in the plasma membrane at the leading edge of a migrating cell, although a more intense staining was observed in the short extensions of the plasma membrane that were formed in some cells at the migration front. Some of the CD9 protein seemed to accumulate in areas close to the nucleus and in areas of the plasma membrane or extensions which were still in contact with other cells. In addition,

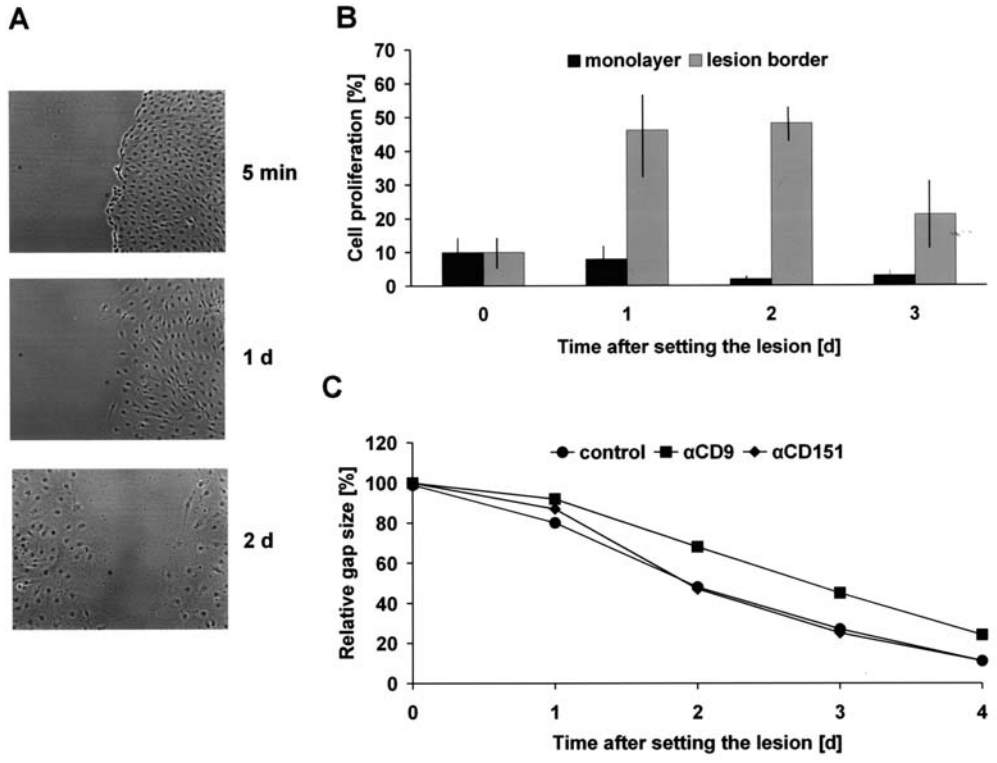

Figure 4. *In vitro* wound healing assay. A lesion was created by scraping off cells from a confluent iBREC monolayer after which the gap was closed in approximately four days. (A) Initiation of gap closure by migration of cells. (B) Proliferation was induced in cells involved in gap closure as measured by BrdU incorporation. (C) Lesion repair was significantly [$p<0.05$ for values at days (d) 2, 3, and 4] inhibited in the presence of an anti-CD9 antibody (αCD9), but not by treatment with anti-CD151 antibody (αCD151) or an isotype matched control antibody.

fragments of the plasma membrane which remained in the gap after scraping as well as fragments that were ripped off and left behind during cell migration were also CD9-positive indicating its expression in adhesive foci. After gap closure, plasma membrane localization of CD9 was re-established in the cells involved, which then were undistinguishable from cells that had persisted undisturbed in the monolayer. Closure of the gap was inhibited by addition of mAb IVA50 against CD9 (Fig. 4C). However, no significant influence of anti-CD9 antibodies on the proliferation rate of cells at the lesion margin or in the monolayer was found (data not shown). Expression of CD63 was detected mainly in intracellular endosomes both in cells at the border and within the monolayer (Fig. 5). Similar to the observed CD151-specific immunostaining detected in confluent and non-confluent iBREC, cells of the confluent monolayer were negative, whereas few of the gap-located cells showed a diffuse intracellular staining that vanished after gap closure (Fig. 5). Closure of the gap as well as the proliferation rate were not influenced by anti-CD151 mAb 11G5a (Fig. 4C; data not shown).

In the absence of fibronectin, the process of gap closure was dramatically retarded, but similarly associated with tetraspanin dynamics. The lesion repair and proliferation rate of cells at the border were not significantly different when iBREC were cultivated in medium supplemented with more (4.5 g/l) glucose to investigate glucose-induced long-term effects.

Inhibition of migration and adhesion of iBREC by treatment with anti-CD9 antibodies. The migration of iBREC towards fibronectin was measured in a modified Boyden chamber assay in the absence or presence of anti-tetraspanin antibodies. As summarized in Fig. 6A, migration of iBREC was strongly inhibited in the presence of mAb IVA50 against CD9 compared to an isotype-matched control IgG. In contrast, migration was not affected by addition of antibodies CC25 or 11G5a binding to the tetraspanins CD63 and CD151, respectively. Although the overall migration rate of iBREC was up to 8 times higher in medium containing 5% serum, the influence of an anti-CD9 antibody on migration was identical (data not shown).

We also measured migration rates of iBREC cultivated in medium containing 4.5 g/l glucose for several weeks. During the first three weeks, the migration rate of these cells was significantly lower than that determined for iBREC cultivated under standard conditions, but with extended cultivation in high-glucose medium; migration rates were readjusted to higher or normal values (Fig. 6B). Inhibition of migration with an anti-CD9 antibody was similarly observed under high-glucose conditions.

To investigate the potential influence of antibodies against tetraspanins on adhesion of iBREC to the ECM component fibronectin, cells were preincubated with the tested antibody and allowed to adhere to the matrix for 1 h. Then an enzyme activity reflecting the number of vital attached cells was measured (Fig. 7). MAb IVA50 against CD9, which was expressed by iBREC mainly in the plasma membrane at cell-cell contact sites and in the spike-like extensions (Fig. 2), strongly inhibited adhesion of iBREC whereas the addition of other anti-tetraspanin antibodies had no effect. When cells were allowed to adhere for 24 h, observed attachment of the

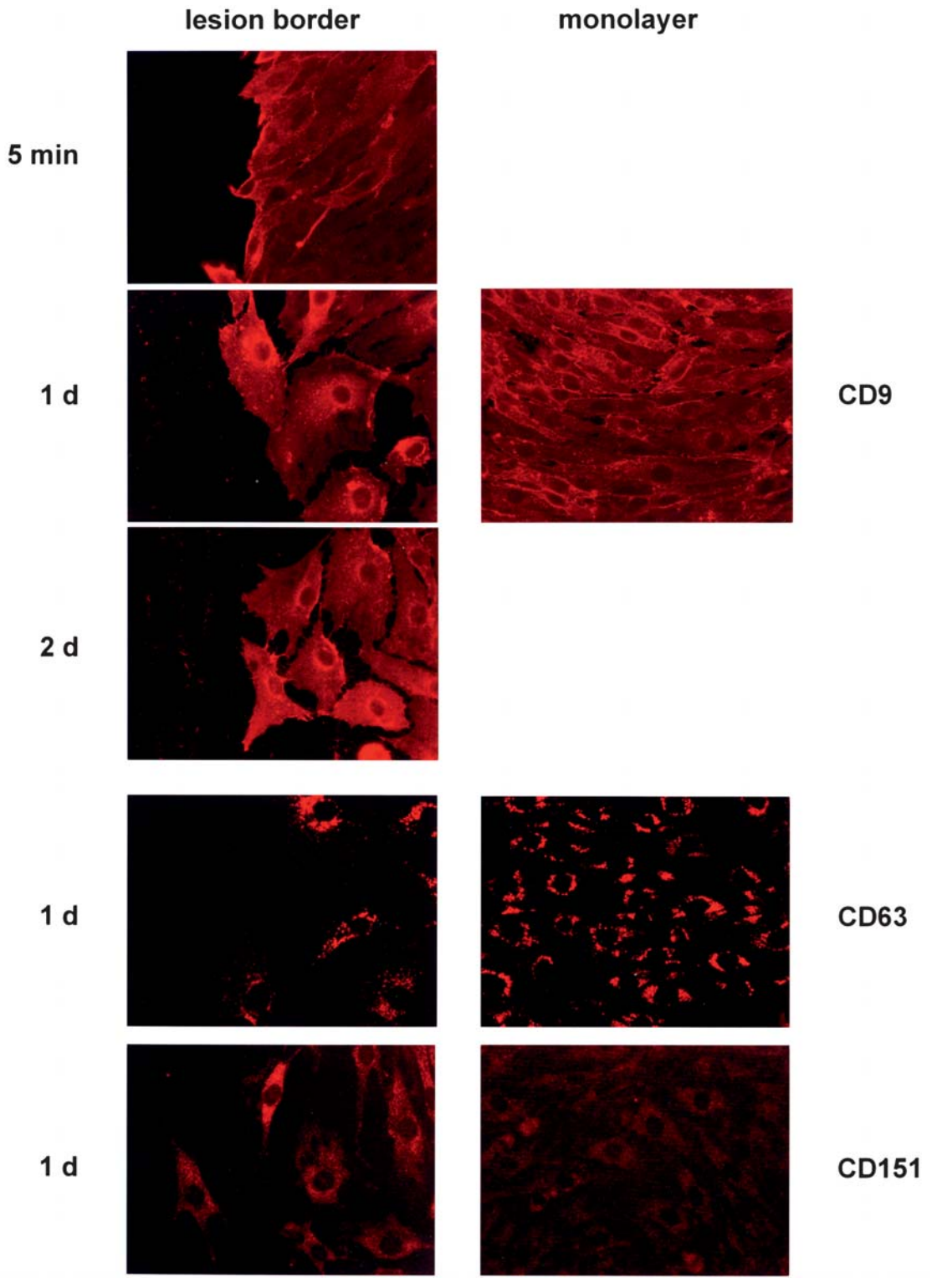

Figure 5. *In vitro* wound healing assay. Expression of CD9, CD63 and CD151 was studied during gap closure by immunofluorescence. Delocalization of CD9 from the plasma membrane and weak re-expression of CD151 was observed within the first 24 h after applying the gap in cells located at the border whereas no change in expression and localization were seen for CD63.

cells was not significantly affected by addition of the anti-CD9 antibody suggesting that it is involved in the initial contact to the matrix rather than in the stabilization of settled cells.

In contrast to migration and adhesion, proliferation of iBREC was found not to be influenced by treatment with any of the antibodies used.

Discussion

The tetraspanin proteins CD9, CD151 and CD63 are expressed in endothelial cells of the macrovasculature (e.g. HUVEC) and have been shown to play a role in wound healing, proliferation, cell migration, adhesion to the extracellular matrix (13,15),

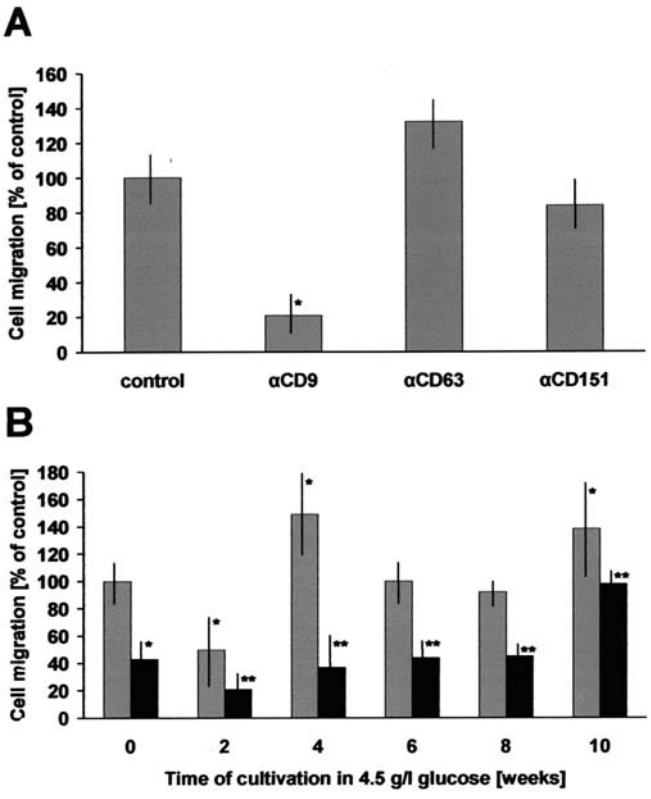

Figure 6. Migration of iBREC measured in a modified Boyden chamber. iBREC were preincubated with the appropriate antibody before seeding on porous membranes. The number of migrated cells was measured after 24 h. (A) Effects of anti-tetraspanin antibodies (αCD9, αCD63, αCD151) on the migration rate. $^{*}p<0.0001$ compared to the control antibody IgG2. (B) The migration rate of iBREC that had been cultivated in medium containing 4.5 g/l glucose (ECGM-hG) in the presence of an isotype-matched control antibody (grey bars) or an mAb specific for CD9 (black bars). $^{*}p<0.005$ compared to the control antibody IgG2 in normal medium; $^{**}p<0.005$ compared to the relevant control antibody IgG2.

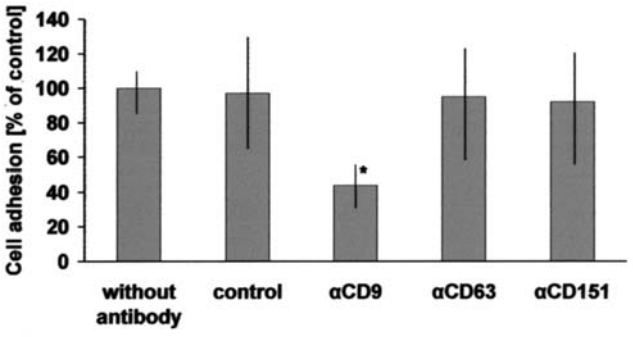

Figure 7. Adhesion of iBREC to fibronectin in the presence of anti-tetraspanin antibodies (αCD9, αCD63, αCD151). The number of vital cells that were attached to the surface after 1 h was measured with an enzyme assay; $^{*}p<0.0001$ compared to the control IgG2.

and intracellular transport (14). Despite the fundamental nature of these processes, tetraspanins expressed by microvascular endothelial cells that are critically involved in the genesis of retinal diseases have not been characterized so far. In this study we identified tetraspanins that contributed to the repertoire of cell surface adaptor and adhesion proteins of retinal endothelial cells and showed experimental evidence for an important function of CD9 in wound healing, cell migration and adhesion to the extracellular matrix in this cell type. In contrast, CD151 and CD63 seemed not to be directly involved in migration or adhesion of iBREC which could be expected in view of their intracellular localization. However, since these proteins seemed to adjust their intracellular locations in distinct compartments in response to environmental changes, their involvement in intracellular trafficking including transport to the plasma membrane, as shown for macrovascular HUVEC (14,24), can be assumed. In an interesting contrast to membrane localization in HUVEC (24,25), CD151 expressed by iBREC is mainly found in the endocytic system pointing to substantial differences between these two subtypes of endothelial cells. However, it cannot be ruled out completely that the different staining patterns result from variable antibody specificity caused by a different context of the recognized epitope (26), although the human and bovine CD151 amino acid sequences are highly homologous.

CD9 is one of the tetraspanins that is strongly expressed by iBREC as confirmed by different analytical methods. In addition to a localization in the plasma membrane, a pronounced CD9-specific intracellular staining was observed. Interestingly, intracellular CD9 was apparently associated with a type of scaffold probably consisting of filaments, as indicated by the clearly visible grid-like structure. Protein relocalization may be an important aspect of modulation of tetraspanin functions which can be concluded from our observation that in the *in vitro* wound healing assay, CD9 delocalized from the plasma membrane of iBREC within hours after creating the gap. As a consequence of the decreased amount of CD9 in the plasma membrane, the cells detached more easily from the fibronectin matrix. The overall process leading to lesion closure, which was weakly inhibited by an anti-CD9 antibody in iBREC, was characterized by coordinated migration and proliferation of initiated cells. The results of our study indicate that CD9 only plays a role in cell migration but not proliferation of iBREC since only the former was significantly inhibited by addition of an antibody against CD9. Interestingly, the responses of HUVEC to treatment with an anti-CD9 antibody included inhibition of migration in a Boyden chamber and in an *in vitro* wound healing assay in combination with a slight stimulation of proliferation, whereas effects on cell adhesion were not observed (13). These differences again confirm that tetraspanin expression and function in microvascular iBREC cannot simply be concluded from previous studies in which HUVEC or other types of endothelial cells were used.

Two-dimensional cell migration is a regulated and coordinated process in which the formation of lamellipodia and filopodia at the leading edge of the migrating cells results in new adhesion points, followed by the contraction of the cell body and detachment of cellular adhesions at the rear end of the cell (27,28). Inhibition of migration as well as adhesion of iBREC to a fibronectin matrix with an anti-CD9 antibody suggests that the functional involvement of this tetraspanin is in the adhesion step of migration. In addition, when cell migration is initiated after setting a lesion in the *in vitro* wound healing assay, CD9 is no longer found in the plasma membrane of cells at the leading edge of the migration front,

indicating that it might also impair cell detachment. Attachment of human primary endothelial cells of retinal origin to fibronectin (29) is dependent on integrin $\alpha_5ß_1$ which is one of the identified potential partners of CD9 in protein complexes in the plasma membrane in other cell types (25). Immunofluorescence studies also confirmed expression of integrins α_5 and $ß_1$ by iBREC (unpublished observation). The appearance of CD9 in intracellular compartments in iBREC also suggests that it could be involved in integrin recycling in migrating cells which has been proposed for integrin-tetraspanin complexes in other cell types (3,30). Further characterization of membrane protein complexes containing CD9 and other tetraspanins will most likely reveal the molecular interactions that are relevant for tetraspanin-dependent physiological functions and their alterations in pathogenic processes in microvascular endothelial cells. Currently, these ongoing analyses are still very much restricted due to the limited number of well-characterized antibodies specific for bovine tetraspanins and potential partners in functional protein complexes.

Interestingly, results of several studies confirmed that antibodies against CD9 can inhibit cellular migration, whereas the total amount of CD9 or the corresponding encoding mRNA usually were inversely correlated with cell motility and associated phenomena, rather suggesting that CD9 is a suppressor of migration (31-33). This might be due to the fact that an antibody only influences plasma membrane-bound CD9 but deregulation of overall expression also effects the intracellular fraction of the protein. In addition, in an *in vivo* situation in which cells are more tightly attached to a complex extracellular matrix than in simplified *in vitro* assays, the role of CD9 in long-term adhesion might be more important than its contribution to cell mobilization, an assumption also in accordance with the frequently found inverse correlation of CD9 expression with invasiveness of malignant cells. Interestingly, we found, in addition to localization in the plasma membrane and in intracellular vesicles, a strong association of CD9 immunoreactivity to an intracellular scaffold in iBREC, suggesting that the observed effects on motility might partly be due to an interaction of CD9 with the cytoskeleton. An indirect modulation of the actin cytoskeleton by CD9 transduction, through downregulation of WAVE-2, has already been shown (34). In addition, members of the EWI protein family have recently been identified as linker molecules of the tetraspanin web to the actin cytoskeleton (35).

Although based on *in vitro* experimentation, our results indicate that CD9 is involved in migration and adhesion of endothelial cells in the microvasculature of the retina. In the pathogenetic mechanisms leading to diabetic retinopathy, the major cause for blindness in adults in western countries, neovascularization is considered a crucial step. Remarkably, the migration rate of iBREC changed during prolonged incubation in elevated levels of glucose in the medium inviting the hypothesis that diabetic conditions may directly affect the behaviour of EC in the retina. To determine the contribution of CD9 and other tetraspanins to pathogenically altered migration of such cells in diabetic retinopathy, further investigations, including analyses of tetraspanin expression in the diabetic human eye, will have to be carried out. In addition, proteins of the tetraspanin family which are involved in migration and adhesion of retinal endothelial cells may provide novel target molecules for therapeutic intervention.

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