Galectin-1 Influences Migration of Retinal Pigment Epithelial Cells

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Purpose. To determine whether the β-galactoside-binding matrix-cellular protein Gal-1 is expressed in human specimens of proliferative vitreoretinopathy (PVR) and to evaluate its influence on RPE migration.

Methods. RT-PCR was used to detect Gal-1-specific transcripts in PVR membranes, and the expression pattern of Gal-1 was examined by immunohistochemistry. Expression of Gal-1 in native, low- and high-density cultured RPE cells was determined by Western blot analysis. Cultured human RPE cells were treated with bFGF, TGF-β2, PDGF-BB, or HGF. The dose-response of Gal-1 mRNA expression was measured by real-time quantitative RT-PCR and Northern blot analysis. Induction of Gal-1 protein was confirmed by Western blot analysis. To study the effect of Gal-1 on RPE migration in vitro, Gal-1 expression was silenced by RNA interference. β-Lactose was used to saturate extracellular galactosides. RPE cell migration was assayed by a modified Boyden chamber assay, with HGF as the chemoattractant.

Results. Gal-1 mRNA expression was present in human specimens of PVR membranes, and staining for Gal-1 was distributed throughout the extracellular matrix (ECM) of PVR membranes. Colocalization was found with laminin and fibronectin and cells of epithelial origin. Western blot analysis revealed greater baseline expression levels in low-density cultured RPE cells than in native and high-density cultured RPE cells. Treatment with HGF caused a dose-dependent increase in Gal-1 expression. Low expression levels of Gal-1 correlated with a reduction of RPE migration to 14% of control. β-Lactose inhibited HGF-induced RPE cell migration to 25% of control.

Conclusions. Gal-1 is present in the extracellular matrix of PVR membranes and may be derived from dedifferentiated RPE cells. The expression level of Gal-1 appears to be related to a migratory RPE phenotype and stimulation by HGF, both conditions implicated in the pathogenesis of early PVR. Furthermore, HGF-induced RPE migration may be dependent, at least in part, on Gal-1- and β-galactoside-dependent mechanisms.

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Proliferative vitreoretinopathy (PVR) is the leading cause of the failure of retinal reattachment surgery. Early PVR is characterized by dedifferentiation, migration, and proliferation of different cells, including fibroblasts and glial and retinal pigment epithelial (RPE) cells at the vitreoretinal interface, giving rise to the formation of contractile fibrocellular membranes on the surface of the neuroretina.1,2 PVR membranes typically contain a predominance of RPE cells, and it is thought that the development of PVR is critically dependent on the proliferative, migratory, and matrix remodeling behavior of these cells.

In the healthy adult eye, the RPE forms a nonproliferating monolayer of polarized, stationary cells that are essential for the maintenance and survival of the photoreceptors.3 After retinal injury, RPE cells become disseminated from their normal site on Bruch’s membrane to multiple loci on the neuroretina and the vitreous. Under the influence of cytokines, such as basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF-BB), and hepatocyte growth factor (HGF), among others,4–7 the dislodged cells are thought to dedifferentiate and exhibit a pseudometaplastic transformation into fibroblast-like cells, which actively divide and migrate.8 These processes are believed to be the key events in the onset of proliferative vitreoretinopathy1,2,8,–10.

Cell migration is a complex biological process that entails sequential adhesion and release from the substrate, a process in which cell–matrix interactions play a key role.11 Although the regulation of cell–matrix interactions is a poorly understood process, it is known that in many instances, cell attachment is mediated by recognition between extracellular matrix (ECM) molecules and transmembrane integrin receptors.12 Recent studies have provided evidence that members of the galectin class of β-galactoside-binding-proteins13,14 also have the potential to mediate cell–matrix interactions by a novel mechanism.15–17 The galectins are a family of soluble lactose-binding lectins characterized by their affinity to β-galactoside moieties.18 They are found on the cell surface and within the ECM, as well as in the cytoplasm and the nucleus of cells.19,20 These proteins recently have attracted increased attention because of their involvement in a large variety of physiological and pathologic processes. Although all members of the galectin family bind to β-galactoside residues, each galectin has a unique fine specificity for more complex oligosaccharides. Therefore, different members of the galectin family may bind to distinct glycoconjugate receptors, resulting in specific downstream effects.21

Galectin (Gal)-1 is one of the most well characterized members of the galectin family and is present with diverse functions in many different cell types and tissues.13,14 Gal-1 can differentially affect cell proliferation.22 It influences adhesion and migration of different cell types23,24 and has been implicated in the induction of apoptosis in activated T-lymphocytes15 and in inhibition of ‘T-cell activation.25 The homodimeric 14.5-kDa protein participates in the interaction of the cell surface with its ECM through binding to the glycoconjugates18 of proteins including laminin,26 fibronectin (Fn),27 vitronectin, and inte-
grins, among others, as well as other biological components. Most of these components have been shown to be present in the ECM of PVR membranes.

When human RPE cells are cultured on plastic, they escape growth arrest and fail to maintain a differentiated morphology. This provides a well-accepted in vitro model for the fibroblast-like phenotype of RPE cells as found in PVR. In a previous study, we have noted an association of RPE dedifferentiation in vitro and upregulation of Gal-1 expression. Given that RPE dedifferentiation is a key event in the pathogenesis of PVR on the one hand and that Gal-1 is upregulated in dedifferentiated RPE and may influence proliferation and migration, we undertook to investigate Gal-1 expression in PVR membranes and to evaluate its influence on RPE migration in vitro.

### Materials and Methods

#### Tissue Samples

Eighteen samples of sub- and epiretinal PVR membranes were obtained from patients who were undergoing vitrectomy surgery for PVR after rhegmatogenous retinal detachment. Operations were performed in the Department of Ophthalmology of the Ludwig-Maximilians-University (Munich, Germany) by different surgeons who used conventional three-port vitrectomy. Ep- and subretinal membranes were separated from the retina by peeling whole tissues. During the operation, membranes were put into phosphate-buffered saline (PBS, pH 7.4) and either snap frozen in liquid nitrogen for mRNA extraction or mounted in optimal cutting temperature (OCT) medium (Merck, Darmstadt, Germany) and stored in liquid nitrogen for cryostat sections.

#### Isolation of Human RPE Cells

Eyes from eight human donors were obtained from the Munich University Hospital Eye Bank and processed within 4 to 16 hours after death. The donors ranged in age from 18 to 79 years. None of the donors had a known history of eye disease. For establishment of human RPE cell cultures, cells were harvested after the procedure, as described previously. Protein lysates from native RPE cells were prepared as described, except that after centrifugation of the RPE cell suspension, the supernatant was replaced with RIPA cell lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS) containing an appropriate amount of protease inhibitors (Complete Mini; Roche, Mannheim, Germany). Further protein extraction was performed as described above. Cell preparations were snap frozen in liquid nitrogen and stored at −70°C for future use.

Methods for securing human tissue were humane, included proper consent and approval, complied with the Declaration of Helsinki, and were approved by the local ethic committee.

#### Human RPE Cell Culture

Retinal pigment epithelial cells obtained from three different donors were grown and characterized as detailed previously. Primary RPE cells of passages 3 to 7 were used for experiments. After reaching confluence, cells were subcultured and maintained in DMEM (Biochrom, Berlin, Germany) supplemented with 10% FCS (Biochrom) at 37°C and 5% CO₂, unless stated otherwise. ARPE-19 cells were obtained from American Type Culture Collection (Manassas, VA), routinely passed once a week, and maintained in DMEM/Ham’s F12 (Biochrom) supplemented with 10% FCS at 37°C and 5% CO₂, as recommended in the vendors’ data sheet. For cell density experiments, cells of the same passage were split at a ratio of 1:4 and allowed to grow to different stages of confluence. RPE cells, which were harvested after they had reached 80% confluence, were referred to as low-density RPE cell cultures, whereas cultured RPE cells maintained in DMEM supplemented with 10% FCS for 4 weeks after they had reached confluence were referred to as high-density RPE cell cultures. For growth factor experiments, cells were grown to 90% confluence, deprived of serum overnight, and subsequently incubated in serum-free medium supplemented with 1, 2, 5, or 10 ng/mL TGF-β2 (R&D Systems, Wiesbaden, Germany); or 10, 50, 100, or 200 µg/mL bFGF (Peprotech, Rocky Hill, NJ); or 10, 20, 40, or 100 ng/mL PDGF-BB (Peprotech); or 10, 20, 50, or 100 ng/mL HGF (R&D Systems). Control cells were incubated under identical conditions but without growth factors in the medium. Each experiment was performed at least three times.

#### Immunohistochemical Staining of Tissue Sections

Seven PVR membranes were cut at a thickness of 8 µm and fixed in 4% paraformaldehyde in PBS. After a wash in Tris-buffered saline (TBS, pH 7.2), Gal-1 antigen was unmasked by placing cryostat sections into boiling 0.01 M citrate buffer (pH 6.0) in a household stainless-steel pressure cooker (Fissler, Iidar-Oberstein, Germany) at 2 bars for 1 to 2 minutes and allowed to cool for approximately 10 minutes before removal from the cooker. Sections were then washed in TBS and preincubated with 3% bovine serum albumin (BSA; Roche, Mannheim, Germany) in PBS for 30 minutes at room temperature (RT) to minimize nonspecific staining. Parallel sections were incubated for 2 hours at RT with mouse anti-Gal-1 (NCL-Gal1; Novocastra, Newcastle, UK), diluted 1:100 in TBS containing 3% BSA. Sections were then washed three times with TBS, followed by an incubation with either rabbit anti-cytokeratin 8 (Biozol, Eching, Germany), or rabbit anti-human glial fibrillary acidic protein (Sigma-Aldrich), or rabbit anti-Fn (Sigma-Aldrich), or rabbit anti-laminin (Sigma-Aldrich) and all antibodies diluted 1:100. Control tissue sections were incubated with BSA-TBS and the secondary antibodies alone. After several washes in TBS, tissue sections were incubated in the secondary antibodies, a Cy-3-conjugated goat-anti-mouse IgG followed by addition of a Cy-2-conjugated swine anti-rabbit IgG from Dianova (Hamburg, Germany) diluted 1:100 in blocking buffer for 1 hour at RT. After three further washes in TBS, cells were mounted in Kaiser gelatin (Merck).

#### Immunohistochemistry of RPE Cell Cultures

For double staining of Gal-1, Fn and laminin in vitro, RPE cells were plated on glass coverslips in 24-well plates and maintained in DMEM supplemented with 10% FCS for 12 days. Cells were fixed and the Gal-1 antigen was unmasked as described earlier. After three washes in TBS and blocking with BSA, specimens were incubated for 15 hours at 4°C with mouse anti-Gal-1 (NCL-Gal1; Novocastra) and rabbit anti-Fn (Sigma-Aldrich), or mouse anti-Gal-1 and rabbit anti-laminin (Sigma-Aldrich; all antibodies diluted 1:100). After several washes in TBS, the secondary antibodies were added as described for tissue sections. For negative immunostaining, control cells were incubated with BSA-TBS replacing the primary antibody.

A fluorescence microscope (Leica, Wetzlar, Germany) was used to visualize the immunofluorescent staining.

#### RNA Isolation and RT-PCR from Tissue Specimens and from Native and Cultured RPE Cells

Total RNA from 11 PVR membranes and RPE cells grown in 10-cm dishes was extracted by the guanidinium thiocyanate-phenol chloroform extraction method (Stratagene, Heidelberg, Germany) and reverse transcribed as described previously. PCR was performed in a total volume of 50 µL using cDNA as a template in the presence of the following primers: Gal-1 sense, 5′-AACCTGGAAGAGTGGCCTTCAG-3′; Gal-1 antisense, 5′-GTAGGTGATTGCCCTCCACCT-3′; product size, 322 bp. EGFR sense, 5′-GGGAGGACTTATCCTCCACACCT-3′; product size, 573 bp. PDGF-BB sense, 5′-GCCAAGGCCTTACGAGGAGG-3′; product size, 573 bp. GADPH sense, 5′-ACATGCCACCTTGGATTTG-3′; product size, 600 bp. GADPH antisense, 5′-GGGATGTTAATCTCCACTCC-3′; product size, 600 bp. GADPH sense, 5′-GCCAAGGCCTTACGAGGAGG-3′; product size, 573 bp. GADPH antisense, 5′-ACATGCCACCTTGGATTTG-3′; product size, 600 bp.
mocyclerc (Mastercycler Gradient; Eppendorf) using PCR conditions detailed previously,32–35 with annealing at 60°C (Gal-1), 55°C (RPE-65), or 55.1°C (GAPDH) for 30 seconds. PCR performed on each sample of RNA that had not been reverse transcribed to cDNA was used as a negative control. For semiquantitative PCR, the number of cycles was optimized by checking amplification after each cycle from cycles 25 to 36 for RPE-65 and from cycles 20 to 34 for GAPDH. This showed that the 30th cycle was in the geometric phase for RPE-65 and GAPDH. RT-PCR results were confirmed by automated DNA sequencing and compared to the expected DNA sequence (Sequivserve, Vaterstetten, Germany). PCR amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide for visualization. The band intensity was measured and quantified (LAS-1000 Imager workstation; RayTest, Pforzheim, Germany; with AIDA software; RayTest). The final amount of PCR product was expressed as the ratio of the RPE-65 gene amplified to that of the GAPDH gene. Experiments were repeated three times.

**Northern Blot Analysis of Galectin-1 mRNA Expression**

Riboprobes for Northern blot analysis were synthesized by PCR, using the same conditions and primer pairs as described for semiquantitative RT-PCR analysis, except that the T7-promoter sequence was added to the 5’ end of the downstream primer. PCR amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide for visualization. Sequences were confirmed by automated DNA sequencing (Sequivserve, Vaterstetten, Germany). After purification with a Qiagen (Hilden, Germany) PCR purification kit, 1 μg DNA was used as a template for in vitro transcription with the digoxigenin labeling RNA kit from Roche. Labeling efficacy was checked by direct detection of the labeled RNA probe with anti-digoxigenin-alkaline phosphatase (Roche). The conditions of Northern blot analysis and chemiluminescent signal detection have been published previously in much detail.32,33 Hybridization was performed with 50 ng/mL digoxigenin labeled Gal-1 specific 322-bp antisense riboprobe. Quantification of the chemiluminescence signal was performed on computer (AIDA software; RayTest). All experiments were repeated at least three times with RPE cells from different cell lines.

**Quantitative Real-Time RT-PCR of Cultured RPE Cells**

Total RNA was reverse transcribed in a 45-μL volume for 1 hour at 42°C containing 9 μL buffer, 2 μL dithiothreitol (DTT; both from Invitrogen-Life Technologies), 0.9 μL 25 mM dNTP (GE Healthcare, Freiburg, Germany), 1 μL RNAse inhibitor (RNAasy; Promega, Mannheim, Germany), and 0.5 μL Microcarrier (Molecular Research Center, Cincinnati, OH). 1 μg random hexamers (2 mg/mL stock; Roche), and 200 U reverse transcriptase (Superscript; Invitrogen-Life Technologies). Quantitative real-time RT-PCR was performed on a sequence-detection system (TaqMan ABI 7700 Sequence Detection System; Applied Biosystems, Weiterstadt, Germany) using heat-activated Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems), as described previously.37 After an initial hold of 2 minutes at 50°C and 10 minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. Commercially available, predesigned reagents (TaqMan; Applied Biosystems) were used for human Gal-1 and 18S rRNA. The mRNA expression was analyzed by standard curve quantification for target and housekeeping genes. All measurements were performed in duplicate. Controls consisting of bi-distilled H2O were negative in all runs. Experiments were repeated three times.

**Protein Extraction and Western Blot Analysis**

Cells grown on 10-cm tissue culture dishes were washed twice with ice-cold PBS, collected, and lysed in RIPA cell lysis buffer. After centrifugation for 30 minutes at 19,000g in a microfuge (5810R; Eppendorf) in the cold, the supernatant was transferred to fresh tubes and stored at -70°C for future use. The protein content was measured using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). Denatured proteins (5 μg) were separated under reducing conditions by electrophoresis on a 5% SDS-polyacrylamide stacking gel and a 15% SDS-polyacrylamide separating gel. After semidry blotting onto a polyvinyl difluoride membrane (Roche), the membrane was blocked with PBS containing 0.1% Tween 20 (PBST; pH 7.2) and 5% bovine serum albumin (BSA) for 1 hour. The primary antibody, a mouse anti-Gal-1 antibody (NCL-Gal1; Novocastra) at a final dilution of 1:100 was then added and allowed to react overnight at RT. After the membrane was washed three times in PBST, an alkaline phosphatase-conjugated swine anti-mouse antibody (diluted 1:20,000; Dianova) was incubated with the membrane for 30 minutes. Visualization of the alkaline phosphatase was achieved using chemiluminescence as described previously.32 Exposure times ranged between 2 and 15 minutes, and chemiluminescent signal quantification was performed on computer (AIDA software; RayTest). All experiments have been repeated at least three times using RPE cells derived from different donors.

**Construction of Galectin-1 Small Interfering RNAs**

Small interfering (si)RNAs were designed according to the recommendations published by Elbashir et al.38,39 Based on the published structures of the human Gal-1 gene, the open reading frame of the targeted Gal-1 mRNA was scanned for 21-nucleotide sequences of the type AA(N19) (N is any nucleotide) and a GC content of less than 50% to obtain a 21-nt sense and 21-nt antisense strand with symmetric 2-nt 3’ overhangs of identical sequence. Selected siRNA sequences were submitted to BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) against the human genome sequence to ensure that only the Gal-1 gene was targeted, as well as to confirm the nonspecificity of the control siRNA. The 21-nt dsRNAs were synthesized by in vitro transcription (Silencer siRNA Construction Kit; Ambion, Huntingdon, UK) according to the manufacturer’s instructions. The targeted Gal-1 mRNA sequence was as follows: 5’-AACCGAUAUCUCAACCCUGGA-3’. A sequence containing the same nucleotides in random order served as nonspecific siRNA control (nonsense mRNA target sequence: 5’-AACCAGUAUCCUAUACCCGA-3’). For in vitro transcription an 8-nt sequence complementary to the T7 promoter primer was added to the 3’ end of the sense and antisense 21-nt nucleotide. Thus, double-stranded siRNAs were created with the
following oligonucleotide templates: Gal-1 antisense, 5'-AACCTGAATCTCACCTGGACCTGTCCTC-3' and sense, 5'-AATCCAGGTTGAGATTCAGGCCTGTCTC-3'; position in gene sequence: 25; nonsense siRNA antisense: 5'-AACCAGTATCCTAATACCGGACCTGTCTC-3'; nonsense siRNA sense: 5'-AATCCGGTATTAGGATACTGGCCTGTCTC-3'.

Oligonucleotides were purchased from Metabion (Munich, Germany). In vitro transcription yielded siRNA at a molar concentration of 21.7 and 32.4 μM, respectively. As confirmed in at least five independent experiments final concentrations of 7.5 nM present in the culture medium gave the best silencing effects.
Cell Culture and Transfection

For posttranscriptional gene-silencing experiments, RPE cells were trypsinized the day before transfection, diluted with fresh medium without antibiotics, and plated on 10-cm dishes to reach 50% to 60% confluence the next day. Transient transfection with siRNAs was performed using reagent (Lipofectin; Invitrogen). To induce siRNA/liposome complex formation, 20 μL of liposomes was preincubated in 0.75 mL serum-free/antibiotic-free DMEM for 5 minutes, whereas siRNA was diluted in 0.75 mL serum-free/antibiotic-free DMEM, giving a final siRNA concentration of 7.5 nM. The two mixtures were combined and incubated for 15 to 20 minutes at RT for complex formation. The reagent complex was then gently distributed over the cells and incubated at 37°C and 5% CO₂. After 6 hours 1.4 mL serum-free/antibiotic-free DMEM was added, and incubation was continued. Control cells were either transfected with a nonspecific duplex containing the same nucleotides in random order or with liposomes and buffer instead of siRNA. After 48 hours, cells were harvested and processed for immunoblotting or trypsinized for the cell migration assay as described. Specific silencing was confirmed by at least five independent experiments.

MTT Assay

The tetrazolium dye-reduction assay (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) was used to test for cell viability after siRNA transfection. The MTT test was performed as described by Mosmann⁴⁰ with some modifications. Cells were transfected and seeded on 10-cm dishes as described earlier. Transfection of cells for migration experiments and MTT assay was performed in parallel with the same cell lines of the same passage. Forty-eight hours after transfection, the cells were washed with PBS, and 2.5 mL of MTT solution (1.5 mL MTT stock, 2 mg/mL in PBS, plus 28.5 mL DMEM) was added, and incubation was continued at 37°C for 30 minutes. The formazan crystals that formed were dissolved by the addition of dimethyl sulfoxide (DMSO, 2.0 mL/dish). Absorption was measured by a scanning multiwell spectrophotometer at 550 nm (Molecular Probes, Garching, Germany). Results are expressed as the mean percentage of untreated control cells. Experiments were performed in duplicate and repeated three times.

Cell Migration Assay

RPE migration was assayed by a modification of the Boyden chamber method⁴¹ in microchemotaxis chambers (NeuroProbe, Gaithersburg, MD) with polycarbonate filters (Nucleopore, Karlsruhe, Germany) with a pore size of 8.0 μm. The filters were coated with laminin (5 ng/cm² in PBS; Sigma-Aldrich) and placed between the chambers. First, the lower half of the chamber was filled with 195 mL DMEM after hepatocyte growth factor (HGF; R&D) at a concentration of 20 ng/mL was added.⁷ RPE cells were trypsinized and resuspended at a concentration of 5 × 10⁶ cells/mL in DMEM supplemented with 0.4% FCS. To investigate β-galactoside-dependent interactions, we preincubated nontransfected cells in medium supplemented with 100 mM β-lactose for 2 hours and resuspended them as just described, but with 100 mM β-lactose in the medium. The cells were then placed in the upper half of the chamber (100 μL/chamber) and incubated at 37°C in 95% air and 5% CO₂ for 4.5 hours. The filters were removed and the RPE cells on the upper side of the filter were scraped off with a cotton tip. The migrated cells on the other side of the filter were fixed in methanol and stained with hematoxylin and eosin. Five randomly chosen fields were counted at a 100× magnification, with a phase-contrast microscope (Leica). Experiments were performed in duplicate and repeated at least three times. Results are expressed as the percentage of the number of untreated control cells.

Statistical Analysis

Statistical analysis was based on the Wilcoxon matched-pairs signed-ranks test and P ≤ 0.05 or less were considered statistically significant.
RESULTS

PVR Membranes

Gal-1 mRNA and Protein Expression. To evaluate whether Gal-1 mRNA is expressed in human PVR membranes, total mRNA from epiretinal and subretinal PVR membranes was extracted and analyzed by RT-PCR. The 322-bp amplicons representing Gal-1 mRNA were present in all investigated PVR membranes (Fig. 1). To determine the spatial distribution pattern of Gal-1 protein within the PVR membranes cryostat sections were stained with an antibody specific for Gal-1. Immunohistochemical staining revealed specific staining in all PVR membrane sections studied (Figs. 2A, 3A). Staining for Gal-1 was present throughout the ECM of the PVR membrane and occurred in a patchy pattern.

Colocalization of Gal-1 with ECM and Cellular Components. Because laminin and Fn are main components of PVR membranes and bind to Gal-1 with high avidity, we sought in the next step to investigate whether there is a colocalization of Gal-1, Fn, and laminin in PVR membranes. Staining for laminin (Fig. 2B) and Fn (Fig. 3B) was present throughout the entire ECM of the PVR membrane. Especially in intensely stained rim areas, the colocalization of Gal-1 and laminin (Fig. 2C) was observed. Immunohistochemical double staining for Gal-1 and Fn showed similar results (Fig. 3C). No differences between epi- and subretinal membranes were found.

To investigate the relationship of Gal-1 to cellular components of PVR membranes, sections were double stained for Gal-1 (Fig. 4A) and GFAP (Fig. 4B) as well as for Gal-1 (Fig. 5A) and cytokeratin 8 (CK8; Fig. 5B). GFAP was used to localize glial cells and CK8 to identify cells of epithelial origin. Whereas no colocalization was present for Gal-1 and GFAP (Fig. 4C), distinct colocalization (Fig. 5C) was found in cells with intensive staining for CK8. These findings suggest that Gal-1 found in the ECM of PVR membranes may be derived from CK8-positive cells. All control sections incubated without the primary antibodies were unstained (data not shown).

Cell Culture of Human RPE Cells

Partial Colocalization of Gal-1 with Fn and Laminin. The next experiments were performed in vitro using primary human RPE cell cultures as a simple in vitro model system for wound repair in PVR.\(^{42-45}\) To find out whether Gal-1 is in the ECM of RPE cells in vitro, cells were cultured for 12 days to allow for ECM deposition. Double-staining experiments for Gal-1 and laminin and Gal-1 and Fn were performed. Gal-1 staining appeared to be homogenously distributed throughout the ECM, with foci of immunoreactivity arranged in threadlike and beadlike patterns (Figs. 6A, 6D). Immunostaining for Fn (Fig. 6B) and laminin (Fig. 6E) revealed a fine extracellular network of immunoreactivity. Double staining of the immunofluorescent images showed partial colocalization of extracellular Gal-1 with Fn (Fig. 6C) and laminin (Fig. 6F). Control cells incubated without the respective primary antibodies were unstained (data not shown).

Synthesis of Gal-1 by Human RPE Cells. PVR membranes typically contain a predominance of proliferative and migratory RPE cells, as well as other cell types at various stages.
of transdifferentiation. To determine whether Gal-1 expression in RPE cells is modulated under these conditions, Gal-1 expression was investigated in native (differentiated) RPE cells and low-density RPE cell cultures, where cells are proliferative and migratory (i.e., the wound repair phenotype), and high-density RPE cell cultures, where cells are more stationary and less motile. Semiquantitative RT-PCR analysis for RPE-65, a microsomal protein that is synthesized by differentiated RPE cells in vivo, was used to evaluate the biochemical differentiation status of the RPE cells (Fig. 7A). In native and high-density cultured RPE cells the 600-bp amplicons representing RPE-65 mRNA were present. Expression in native RPE cells was two times higher when compared with high-density cultured RPE cells. No RPE-65 transcript was detectable in low-density cultured RPE cells.

Immunoblot analysis for Gal-1 revealed a single band of approximately 14.5 kDa in size (Fig. 7B), in both native and cultured human RPE cells. Whereas in native, stationary RPE cells only a very faint band was detected, low-density cultured RPE cells showed a 2.0-fold higher amount of the 14.5-kDa protein when compared with the native RPE cells. Cultured RPE cells that had been allowed to grow to high density in culture showed a significant decrease in Gal-1 expression similar to native RPE cells. A representative blot is depicted in Figure 7B. These results suggest that Gal-1 is upregulated in proliferating, dedifferentiated cultured RPE cells and that the level of Gal-1 expression may be linked to cell density, but also to the differentiation state of RPE cells.

**Effect of Growth Factors on Gal-1 Expression.** In the next step, we sought to determine whether serum-derived cytokines implicated in the pathogenesis of early PVR may lead to an increased expression of Gal-1. In an attempt to reproduce pathologic conditions we chose to study the expression of Gal-1 after incubation of RPE cells with an assortment of cytokines that have been found at elevated levels in the vitreous of patients who have PVR. To screen for dose dependence of Gal-1 expression after growth factor treatment, we treated cultured RPE cells with 0, 1, 2, 5, or 10 ng/mL TGF-β; or 0, 10, 20, 40, or 100 ng/mL PDGF-BB; or 0, 10, 50, 100, or 200 pg/mL bFGF; or 0, 10, 20, 50, or 100 ng/mL HGF, respectively. The changes in Gal-1 mRNA expression after growth factor treatment were quantified by real-time quantitative RT-PCR (for TGF-β, PDGF-BB, and bFGF) as depicted in Figure 8. Dose dependence of Gal-1 expression after HGF treatment was quantified by Northern blot analysis. A representative blot is shown in Figures 9A and 9B. Whereas different doses of TGF-β, PDGF-BB, and bFGF had no significant effect on Gal-1 mRNA expression (Fig. 8), expression levels of Gal-1 increased dose dependently by 1.3- to 2.7-fold after treatment with HGF (Figs. 9A, 9C). To validate that this increase in mRNA transcription translates into increased protein synthesis, whole cellular protein extracts were analyzed by Western blot analysis after treatment of cells with the respective cytokines at doses that had been shown to be relevant in PVR. Treatment with HGF increased the amount of Gal-1 in cultured RPE cells approximately twofold (P < 0.05). Treatment of the cells with...
the other cytokines had no effect on Gal-1 protein expression (Fig. 10).

**Effect of Silencing of Gal-1 Expression on RPE Migration**

HGF has been shown to be a strong chemoattractant for cultured RPE cells. In an attempt to evaluate a possible functional role of Gal-1 in HGF-mediated RPE migration, we specifically suppressed Gal-1 expression in cultured RPE cells by posttranscriptional gene silencing through RNA interference and analyzed RPE migration. Forty-eight hours after transfection, gene silencing was documented by Western blot analysis (Fig. 11A). Typical of siRNA technology, a substantial downregulation of Gal-1, but not a complete knockout, was achieved when compared with the control. Posttranscriptional gene silencing with Gal-1 siRNA reduced Gal-1 expression to 27% of the expression level of the untreated control. Gal-1 mRNA expression levels in control transfected cells remained unchanged.

RPE migration was assessed using microchemotaxis chambers with HGF as chemoattractant. Forty-eight hours after Gal-1 silencing, the migration of RPE cells with low expression levels of Gal-1 showed 10% to 20% (P < 0.05) of migratory activity when compared with the untreated control, whereas cells transfected with liposomes and buffer alone achieved 100% to 124% of migratory activity of the control cells (Fig. 11B). Nonsense siRNA induced a slight reduction of RPE migration, β-Lactose decreased RPE migration activity to levels of 20% to 30% (P < 0.063) compared with untreated control cells (Fig 11B). To rule out that decreased RPE migration results from reduced cell viability after transfection, cell survival was assessed in parallel experiments by MTT conversion. In cells transfected with liposomes and buffer alone, cells transfected with nonsense siRNA and Gal-1 siRNA, MTT conversion rates remained at 86% to 115% of the untreated control, indicating that survival was not greatly affected by the transfection procedure (Fig. 11B). These results suggest that the observed slight reduction of migration in RPE cells transfected with nonsense siRNA may most notably have been due to an effect of the transfection procedure leading to impairment at the functional level, but not to decreased cell viability.

**DISCUSSION**

The present study demonstrates for the first time that Gal-1 mRNA and protein are present in the ECM and cellular components of PVR membranes. In vitro, dedifferentiation of RPE cells increased Gal-1 expression, which was further enhanced on treatments with HGF, a growth factor known to be increased in the vitreous of patients with PVR. Functionally, low expression levels of Gal-1 induced by RNA interference, corresponded with reduced RPE migration in response to HGF. Furthermore, RPE migration was reduced in the presence of...
lactose, suggesting that RPE migration may, at least in part, be dependent on Gal-1 and the carbohydrate-binding domains of galectins.

In our immunohistochemical analysis, we noted an association between Gal-1 and RPE cells in PVR membranes, an observation that is consistent with the notion that RPE cells, both in vivo and in vitro, have the ability to synthesize Gal-1.25,31 Gal-1 was distributed throughout the ECM of the PVR membrane. Moreover, our in vitro data show that some of the protein was secreted and deposited in the ECM, consistent with a proposed role for Gal-1 in ECM remodeling during wound healing.49,50 Laminin and Fn are main components of the ECM found in PVR membranes, and we found a partial colocalization with extracellular Gal-1, both in PVR membranes and in the ECM deposited by RPE cells in culture. This may be of particular importance, because in its function as a matricellular protein, Gal-1 binds to laminin and Fn with high affinity, thereby mediating the cross-talk between cell surfaces and binding to the ECM molecules.23 Matricellular proteins, such as thrombospondin, SPARC, and tenasin, have been proposed to spawn the environment necessary to permit proliferation and migration of RPE cells in early PVR.6 Gal-1 shares some features of these matricellular proteins and is a key regulator of a variety of cellular activities, including proliferation, migration, and differentiation of cells. Extracellular Gal-1 forms networks of complementary glycoconjugates on the cell surface that modulate the cell response, and locally produced Gal-1 may modulate cellular behavior during tissue repair.53,26,49 An increase in Gal-1 expression is associated with a considerable number of pathologic conditions, in which migration and proliferation of cells are assumed to be causative factors. Elevated expression levels of Gal-1 have been reported in atherosclerosis28 and liver fibrosis,51 and it has been thought to correlate with invasive characteristics and a poorly differentiated phenotype in tumor cells.52,53

There is little information on regulation of the Gal-1 gene in the literature. The expression changes during development,54 and it has been shown that the Gal-1 promotor is regulated by the level of DNA methylation in several cell types, but Gal-1 expression in human RPE cells has not been studied.55 Western blot analysis revealed the presence of a basal level of Gal-1 in native, differentiated RPE cells of normal human donors. Also, in high-density cultured RPE cells, where cells are densely packed and proliferation and migration are less marked, only low expression levels of Gal-1 were detectable. This was in clear contrast to low-density RPE cell cultures, where cells are isolated and migratory and is consistent with findings made in vascular smooth muscle cells, where Gal-1 is upregulated after proliferation.28 However, upregulation of matricellular proteins in growing cells may not be a general phenomenon. For example, synthesis of SPARC, another matricellular protein implicated in PVR pathogenesis, was increased in high-density RPE cell cultures.44 The detection of low Gal-1 expression levels in both, high-density RPE cell cultures and native RPE cells, suggests that expression of Gal-1 changes with cell density and the differentiation state of the cells, and that it may be related to a proliferative, dedifferentiated RPE phenotype. These results further lead to the assumption that serum stimulation and dedifferentiation of RPE cells as found in PVR disease may contribute to an increase in Gal-1.

The factors responsible for induction of Gal-1 expression are not known. In vascular smooth muscle cells TGF-β, PDGF, and angiotensin II have been discussed as possible mediators of Gal-1 expression.56,57 We have now shown that low concentrations (5-10 ng/ml) of HGF (a potent mitogen for RPE cells) induce a significant increase in Gal-1 mRNA and protein expression in RPE cells (Figures 9 and 10). This increase was not a result of cell proliferation, as we were able to show that the HGF-induced increase in Gal-1 expression was independent of changes in cell number (data not shown).

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for the observed increase of Gal-1. Also, activation of human endothelial cells by TGF-β, IFNγ, and IL1 increased Gal-1 expression in these cells. However, as opposed to our findings the upregulation of Gal-1 in these studies was only moderate. To the best of our knowledge this is the first study to demonstrate that HGF-induced upregulation of Gal-1 synthesis is, at least in part, due to increased production of the protein rather than decreased degradation. Gal-1 can influence several cellular functions including repair-related cellular activities such as proliferation, migration, and differentiation. HGF has mitogenic, motogenic, and morphogenic activities when cultured with epithelial cells. Increased expression of HGF and its receptor, c-Met, is present within the stromal cells of cellular PVR membranes, and HGF is increased in the vitreous of patients with PVR. In cultured RPE cells, HGF acts as a modest mitogen, but has been shown to be a potent chemotactant. Only little is known about the mechanisms involved in HGF-induced RPE migration. It has been shown that one aspect may be the loss of intracellular junctions, as well as activation of the MAP kinase pathway and changes in gene expression of β-catenin. Given the possible roles of Gal-1 and HGF in RPE migration, we questioned whether HGF-induced Gal-1 may be linked to RPE cell migration. In our in vitro experiments, we demonstrated that RPE cells containing low amounts of Gal-1 showed reduced RPE migration in response to HGF. This was in contrast to control cells with baseline expression of Gal-1. Furthermore, to evaluate the role of extracellular galectins and the β-galactoside-dependence of the process extracellular galectin was blocked by β-lactose; and, indeed, in the presence of β-lactose, a decrease in RPE migration was observed. This is consistent with the notion that Gal-1 may play a role in RPE migration and that the carbohydrate binding domain may be involved in this process. However, at present, more than 10 galacten have been isolated. The Gal-2, -5, and -7 exhibit a restricted distribution, whereas Gal-1, -3, -8, and -9 exhibit a broad tissue distribution. Among these, Gal-3 and -7 have been reported to play a role in the re-epithelialization of corneal wounds, and expression of both, Gal-1 and -3, has been found in the outer plexiform layer; outer limiting membrane, and the RPE in bovine, rat, and mouse retinas. To date, there are no further data regarding the presence of other members of the galectin family and their respective functions in the human RPE. Although RPE migration was clearly reduced in cells with low Gal-1 expression levels, we cannot rule out the possibility that other galectins or β-galactoside-binding proteins may play a role in RPE migration. Clearly, further studies are needed to investigate this concept.

Caution is needed in applying these in vitro results to the clinical and pathologic situation. In vivo there are many other ECM molecules but Gal-1 and laminin, and precise effects on cell behavior critically depend on the molecular balance among Gal-1, ECM molecules, and cytokines in the microenvironment of the cell. Nevertheless, we have identified Gal-1 as a new component of the ECM of PVR membranes and found that it is upregulated on RPE dedifferentiation and stimulation with HGF, both pathologic conditions implicated in PVR. Our data further suggest that Gal-1 can profoundly affect RPE migration under these conditions.

We believe that these mechanisms may have relevance in the pathogenesis of early PVR. At the onset of PVR, RPE dedifferentiation, together with an HGF-mediated increase of Gal-1 expression may help RPE cells to migrate on laminin-containing matrices, which may ultimately contribute to the formation of tractional fibrocellular sub- and epiretinal membranes. Although fully speculative at this point, the inhibition of β-galactoside-binding proteins may offer a future therapeutic approach to prevent migration of detached RPE cells.

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References


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