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Molecular imaging system for possible prediction of active retinopathy in patients with *Diabetes mellitus*

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Summary. *Objective*: The deposition of advanced glycation end products is enhanced in *Diabetes mellitus* (DM) and has been linked to diabetic complications such as a microvascular disease. Glycated proteins have receptors on mononuclear blood cells (MBCs) and have been shown to generate reactive oxygen species altering gene expression and modifying cellular targets, such as endothelial cells. Retinal angiopathy is a frequently observed microvascular complication in DM-patients. Because of the central role of activated MBCs, we hypothesised a functional link between specific alterations in gene expression of MBCs, an increased activity of matrix proteases in serum, and the extent of retinal angiopathy in DM.

Material and methods: An appearance and proliferation index of diabetic retinopathy was evaluated in 38 DM-patients using fluorescein angiography. Alterations of gene transcription levels in MBCs were investigated using hybridisation of individual mRNA-pools to Atlas Array with a concomitant quantification of specific cDNAs by "Real-Time"-PCR. The activity of matrix metalloproteinases MMP-2 and MMP-9 in individual serum samples was measured by zymography combined with densitometric imaging system.

Results and conclusions: Hybridisation to Atlas Array of mRNA-pools isolated from MBCs revealed an enhanced expression of recoverin in DM-patients compared to the control group. "Real-Time"-PCR showed the highest recoverin levels in the DM-subgroup with a high proliferation index. MMP-2 activity was highly increased in 36% of all patients, and in 44, 44, and 19% of patients with proliferative retinopathy, advanced proliferative retinopathy, and no detectable proliferation respectively. In those 3 groups MMP-9 activity was highly increased in 56, 67, and 31% of patients respectively, and in 44% of all DM-patients. In contrast to patients with active proliferation, the simultaneous high activation of all three genes was not observed in patients without active proliferation. The *ex vivo* molecular imaging system developed in this work may be helpful for the prediction of active proliferative retinopathy in DM.

Keywords: Diabetic retinopathy – Molecular Imaging – Differential gene expression – Recoverin – Metalloproteinases – Smoking

Introduction

Diabetic retinopathy is a leading cause of visual loss. A microvascular disorder has been implicated in the pathomechanisms of the disease (Ogura, 2000; Boeri et al., 2001), and it can be one of the earliest events observed even in a very young diabetic population (Krakoff et al., 2001). Abnormalities in the microcirculation observed in DM-patients correlate significantly with the severity of hyperglycemia (Service and O'Brien, 2001). However, both the occurrence and the severity of diabetic microvascular disease vary among individual patients and do not necessarily correlate with the duration of DM (Cheung et al., 2001). As yet, the molecular mechanisms influencing the severity of diabetic microvascular disease are not fully understood.

Diabetic retinopathy has an underlying inflammatory component, manifesting itself in leukocyte recruitment and up-regulation of genes responsive to inflammatory processes (Joussen et al., 2002). Adhesion of leukocytes to the retinal vasculature is one of the earliest events in experimental DM that results in breakdown of the blood-retinal barrier, endothelial cell damage, and capillary non-perfusion (Ogura, 2000; Boeri et al., 2001; Hofman et al., 2001). Both ICAM-1 and the leukocyte integrin CD18 have been shown to be up-regulated in diabetic retinopathy (Joussen et al., 2002). By blocking adhesion molecules on the vascular endothelium, the leukocyte-endothelial interactions and concomitantly a retinal atrophy were effectively inhibited in animal models (Ogura, 2000).

Considering inflammation as one of the important factors in diabetic retinopathy, we analysed a differential gene expression in mononuclear blood cells (MBCs), hypothesising a possibility to select molecular markers specifically expressed during progression and possibly even

during development of the disease. The identification and study of novel genes involved in retinal angiogenesis may also define new targets to suppress retinal neovascularisation in DM. Therefore, we performed a comparative investigation of gene expression in MBCs isolated from DM-patients with and without active retinopathy. In addition, we measured the activity of two matrix metalloproteinases (MMP-2 and MMP-9) in the serum of our patients, since an activation of these genes is known to play a key-role in tissue remodelling and neovascularisation and is supposed to be involved in vascular pathomechanisms conditioned by hyperglycemia (Uemura et al., 2001; Mene et al., 2001).

Material and methods

Subjects

38 individuals with DM were recruited for this investigation. The diagnosis of DM was based on a detailed clinical history and biochemical examination of the recruited persons. All investigations conformed with the principles outlined in the Declaration of Helsinki and were performed with permission from the Ethic's Committee of the Medical Faculty, University of Bonn.

Ophthalmologic examination

Patients that came to the Department of Ophthalmology for evaluation of diabetic retinopathy and that were subsequently scheduled for fluorescein angiography were asked to participate in this study. Informed consent was obtained, and multiple data regarding DM as well as details possibly affecting the patient's general health status were recorded. Before angiography, approximately 20 ml of full blood anti-coagulated with K_3EDTA were drawn and delivered immediately for molecular biological examination. All patients received a full eye exam.

Isolation of mononuclear blood cells

Blood samples (20 ml) anti-coagulated with K_3EDTA were collected from DM-patients (see clinical data) and non-diabetic individuals (clinical data are not shown). Leukocytes were separated using Ficoll-Histopaque gradients (Histopaque 1077, Sigma, USA) as described previously (Golubnitschaja-Labudova et al., 2000).

Isolation of total RNA, mRNA and first-strand-cDNA synthesis

Isolation of total RNA from aliquoted samples of mononuclear blood cells and the concomitant mRNA isolation was performed using RNAzol™ B (WAK-Chemie Medical GmbH, Germany) and Oligotex mRNA Mini Kit (Qiagen, Germany) respectively, according to the protocols supplied by the manufacturers. cDNA synthesis was performed using the "First-Strand cDNA Synthesis Kit" (Amersham Biosciences, UK). For each cDNA synthesis, 1 μg mRNA was reverse transcribed using an oligo$(dT)_{18}$ primer in a final volume of 15 μl each, according to the protocol supplied by the manufacturer.

Hybridisation to "Atlas Array"

A) Preparation of biotin-labelled cDNA probes

100 ng of cDNA were labelled using a SpotLight™ Random Primer Labelling Kit (BD Biosciences Clontech, USA). Template cDNA together with 5 μl of 10× Random Primer Mix were heated to 97°C for 3 min in a final volume of 31 μl and chilled quickly on ice. After adding of the reaction mix (5 μl 10× Klenow Reaction Buffer, 5 μl 10× Klenow Labelling Mix, 1 μl Klenow Enzyme and 8 μl ddH_2O) the labelling reaction was performed at 37°C for 30 min. Then the reaction was stopped by adding 2 μl of 0.5 M EDTA (pH 8.0).

B) Purification of biotin-labelled probe

Unincorporated biotin-labelled nucleotides and small (<0.1 kb) cDNA fragments were removed using the NucleoSpin Extraction columns according to the protocol supplied by the manufacturer (BD Biosciences Clontech, USA). The concentration of the newly synthesised biotin-labelled probes was determined by UV spectroscopy. Finally, the biotin-labelled probes were stored at −20°C until hybridisation to Atlas Array.

C) Hybridisation of biotin-labelled probes to Atlas Array

For the hybridisation, Atlas Human 1.2 Array (Cat. #7850-1, BD Biosciences Clontech, USA) and SpotLight™ Chemiluminescent Hybridisation & Detection kit (BD Biosciences Clontech) were used. Each Atlas Array (membrane) was wetted by placing it in a dish of de-ionised H_2O, transferred to the hybridisation bottle and pre-hybridised in the hybridisation mix at 42°C for 3 hours before hybridisation. Each hybridisation was performed overnight at 42°C with individual biotinylated cDNA probes. These probes were denatured in 100 mM NaOH at 68°C for 20 min and neutralised with 0.5 M NaH_2PO_4 (pH 7.0) at 68°C for 10 min before the hybridisation in the Hybridiser (Techne, UK).

D) Stringency Washes

Hybridisation solution was discarded and the membranes were washed 4-times in 200 ml of Wash solution 1 (2 × SSC and 1% SDS) for 30 min at 60°C with continuous agitation. Wash solution 1 was then replaced with Wash solution 2 (0.1 × SSC and 0.5% SDS), and the membranes were washed two more times for 30 min at 48°C.

E) Probe detection and signal visualisation

The membranes were incubated first in 25 ml Blocking Buffer per Atlas Array at room temperature for 1 hour before the incubation with Streptavidin-HRP Conjugate (final dilution of 1:300) for 1 hour with constant gentle agitation. Afterwards the membranes were washed 4-times in 1× Wash Buffer (SpotLight™ Chemiluminescent Hybridisation & Detection kit, BD Biosciences Clontech) for 10 min with concomitant equilibration in Substrate Equilibration Buffer (kit) for 5 min at room temperature before incubation in 8 ml Working Solution (the mix of Luminol/Enhancer Solution and Stable Peroxide Solution, supplied in the kit) per membrane for 5 min. After removing excess liquid the Atlas Arrays were processed for auto-radiography with exposure times of 1, 2, 5, 10 and 30 min. The exposed spots were further scanned and analysed with AtlasImage software (BD Biosciences Clontech).

Reverse transcriptase PCR (RT-PCR) and real-time quantitative PCR (RT-QPCR)

In order to detect the expression of recoverin in leukocytes and to optimise the reaction conditions for Real-Time Quantitative PCR, reverse-transcriptase PCR was performed using specific primer-sets designed to β-actin (accession number BC014861, positions 203–221 and 411–394 bp in mRNA) and recoverin (accession number S43855, positions 189–207 and 498–479 bp in mRNA). The synthesised oligonucleotides had the following sequences: for β-actin -5′GATGGTGGGCATGGGTCAG3′ and 5′TGGGGTTCAGGGGGGCCT3′, and for recoverin -5′TGGGG ACAGCAAAAGTGG3′ and 5′CACTCCAGCTTCTGGTTGG3′ used as forward and reverse primers respectively. cDNA synthesis was performed

using the "First-Strand cDNA Synthesis Kit" (Amersham Biosciences, UK). The PCR mixture contained 1 × PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris, pH 8.8, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol), dNTPs (each at 1.25 mM), primer pairs (100 pM each per reaction), and 10 ng of cDNA-template in a final volume of 50 μl. Reactions were hot-started at 95°C for 5 min before adding 1.5 units of Taq Polymerase (Red-Hot®, ABgene, UK) at the annealing temperature of 56°C followed by polymerisation at 72°C for 1 min. Amplification was carried out in a Perkin Elmer "DNA Thermal Cycler TC480" for 45 cycles (denaturation for 45 sec at 95°C, annealing for 45 sec at 56°C, and polymerisation at 72°C for 30 sec), followed by a final 7 min extension at 72°C. Negative controls without DNA as well as positive controls with a sequenced template were performed for each set of PCR experiments. PCR products (50 μl) were directly loaded onto 3% agarose gels ("Wide Range"-Agarose for analysis of DNA fragments longer than 50 bp, Sigma), stained with ethidium bromide after electrophoresis, directly visualised under UV illumination, and imaged using a specialised imaging system (MWG-Biotech, Germany). The specificity of each PCR amplification was controlled using the site specific restriction analysis of target PCR products. The amplification products underwent an extraction from the agarose gel using DNA isolation kit (DNACleanTM, Hybaid-AGS GmbH, Germany) before digestion. They were digested in a final volume of 50 μl with 20 units of each restriction endonuclease for 4 hr, according to conditions specified by the manufacturer (Roche, Switzerland), and imaged after electrophoresis (MWG-Biotech, Germany).

In order to profile precisely the changes in an expression of recoverin, RT-QPCR was used. SYBR® Green I (Molecular Probes, USA) was utilised as the intercalation dye and fluorescent reporter molecule detecting the accumulation of the amplified double-stranded product in the iCycler iQIM Detection System (Bio-Rad, USA) according to the protocol supplied by manufacturer. 50 ng of the synthesised cDNAs (see RT-PCR) were used for each real-time PCR analysis. The reaction mixtures had the same contents as for RT-PCR with an exception of Red-Hot® polymerase (ABgene, UK) which has been substituted for Taq DNA polymerase (Roche, Switzerland) in order to avoid colour signal disturbances. The same amplification program has been used in both qualitative RT-PCR and quantitative real-time PCR analysis. The algorithm of the iCycler iQIM Detection System normalises the reporter signal (non-intercalated SYBR® Green I) to a passive reference and multiplies the SD of the background signal in the first few cycles by a default factor of 10, to determine a threshold. The cycle at which this baseline level is exceeded is defined as threshold cycle (C_t). C_t depends on the initial template copy number and is proportional to the log of the starting amount of nucleic acid (Heid et al., 1996). By subtracting the difference of the C_t values of recoverin from those of the housekeeping gene (β-actin), the data have been normalised. The relative levels were calculated for each sample based on the differences in C_t values (Heid et al., 1996).

Zymography

For determination of gelatinase activity of MMP-2 and MMP-9 in blood serum "Ready-Gelatin-Gels" (Bio-Rad, USA) were used according to the instructions of the manufacturer. Two microliters from individual serum samples were electrophoresed under non-reducing conditions using Criterion™ Precast Gel System (Bio-Rad, USA). After electrophoresis, each gel was incubated at room temperature in 2% Triton X-100 for 2 × 30 min in order to remove the traces of sodium dodecyl sulphate, and then incubated overnight at 37°C in buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, containing 5 mM $CaCl_2$ and 0.02% NaN_3). Afterwards a staining with 0.5% Coomassie blue G-250 (Sigma, USA) was performed for each gel. The proteolytic activity of each gelatinase (A and B) was identified as a clear band on a blue background according to the correspondent molecular weight of each gelatinase (A and B). Gels were dried between cellophane sheets with a GelAir™ Drying System (Bio-Rad, USA) and then scanned with a yellow filter using Adobe Photoshop (Adobe System, USA) in grey-scale mode. Densitometric analysis of zymographic lysis zones at 66 and 86 kDa for gelatinases A and B respectively was performed using "Quantity One" imaging system (Bio-Rad, USA).

Statistical evaluation

Statistical significance was calculated by the two-sided unpaired Student's t-test and considered at the $p < 0.05$ level.

Results

Clinical data

DM-patients were 59.8 ± 9.9 years old (see Table 1). From 38 patients, 35 subjects suffered from DM type 2, and 3 subjects from DM type 1. 76% of all patients were insulin dependent. 34% were smokers. DM duration varied considerably from 3 to 43 years. Therefore, 3 subgroups A, B, and C with a DM-duration of 3–9, 10–19, and more than 20 years respectively, were selected. An active proliferative retinopathy was found in exactly 50% of all DM-patients (with a male to female ratio of about 1:1, the mean age being 60.7 ± 7.9 years). There were 50, 45, and 56% of patients with proliferative disease in subgroups A, B, and C respectively. The most prominent proliferations (noted as "++", "+++" and "++++" in Table 1) were observed in 28% of all DM-patients, and in 33, 9, and 37.5% in subgroups A, B, and C respectively. Proliferative disease, marked proliferative disease and no active proliferative disease was observed in 58, 25, and 42% of non-smokers and in 42, 33, and 58% of smokers respectively. In our study, no correlation between individual HbA1c (an indicator for long term blood glucose level) values and the occurrence or degree of proliferative retinopathy was found.

Hybridisation of cDNAs to Atlas Array

The hybridisation of labelled cDNAs to Atlas Array revealed a differential expression of 87 genes in mononuclear blood cells of the DM-group which were either induced or suppressed compared to that of a group with non-diabetic individuals without retinopathy. Among these genes we found a highly induced expression of recoverin in mononuclear blood cells isolated from DM-group (Fig. 1).

RT-PCR of recoverin in cDNA samples

RT-PCR of both β-actin and recoverin cDNA fragments demonstrated the specific amplification of the target PCR products with the expected length of 209 and 309 bp

Table 1. Information about the patients grouped according to the DM-duration and about the parameters measured; HbA1c shows values of long term blood glucose levels (normal values are 5.0–6.9); APT indicates any type of anti-proliferative therapy

No. of patient	Age	Sex	DM-type	DM-duration	HbA1c	Insulin dependence	APT	Nicotin/ years	Proliferation
Subgroup A with the DM-duration 3–9 years (n = 10), 58.5 ± 8.2 years old									
34	59	M	DM 2	3	11.3	−	+	+40	No data
56	65	M	DM 2	3	7.5	−	−	+10	−
11	51	M	DM 2	4	6.8	−	+	+8	++
33	47	F	DM 2	5	8.5	+	−	−	−
44	57	M	DM 2	6	8.2	−	−	+40	+
16	68	M	DM 2	6	5.3	+	+	−	+
17	66	M	DM 2	7	6.5	+	+	−	+++
55	49	M	DM 2	8	6.4	+	+	+40	++
37	54	F	DM 2	8	6.0	−	−	−	−
6	69	M	DM 2	9	8.7	−	−	+40	−
Subgroup B with the DM-duration 10–19 years (n = 11), 59.4 ± 5.9 years old									
38	56	M	DM 2	10	7.0	+	−	+4	−
24	63	M	DM 2	12	6.5	+	−	−	+
20	65	M	DM 2	12	6.2	−	−	+7	−
36	63	M	DM 2	13	7.3	+	+	−	+
27	57	F	DM 2	14	7.7	+	+	−	+
1	61	M	DM 2	15	9.0	+	−	−	−
50	49	F	DM 1	18	5.3	+	−	+35	−
41	53	M	DM 2	18	7.7	+	+	−	−
46	67	F	DM 2	18	11.0	+	+	−	+
26	54	F	DM 2	19	10.7	+	+	−	++
30	65	M	DM 2	19	6.1	+	−	+18	−
Subgroup C with the DM-duration of DM more than 20 years (n = 17), 60.7 ± 12.8 years old									
8	38	F	DM 2	20	8.6	+	+	+5	+++
32	62	M	DM 2	20	9.1	+	+	−	++++
31	65	F	DM 2	20	10.5	+	−	−	+
5	67	F	DM 2	20	6.3	+	+	−	++
18	23	F	DM 2	23	6.1	+	+	−	−
43	65	M	DM 2	23	6.3	+	+	No data	−
15	67	M	DM 2	23	5.8	+	+	−	+
7	63	M	DM 2	25	7.7	+	−	−	+
25	67	M	DM 2	27	8.1	+	+	−	−
29	67	M	DM 2	29	No data	+	+	−	No data
2	65	M	DM 2	30	6.6	+	+	−	++
13	66	M	DM 2	31	6.4	−	+	+40–50	++
28	46	F	DM 1	33	6.8	+	+	+1	−
23	66	F	DM 2	33	6.5	−	+	−	++
40	70	M	DM 1	38	7.0	+	−	−	−
19	66	F	DM 2	39	7.2	+	+	−	−
51	70	F	DM 2	43	7.8	+	+	−	−

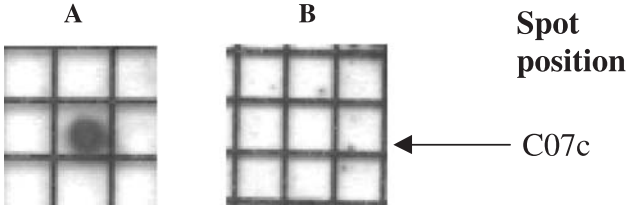

Fig. 1. Expression profiling of recoverin by hybridisation of the pooled cDNAs samples isolated from DM-patients (**A**) and non-diabetic individuals without retinopathy (**B**)

respectively that was in addition confirmed by the site specific restriction analysis (Fig. 2).

Recoverin expression in mononuclear blood cells

The results of the recoverin quantification are shown in Table 2. Based on the relative C_t values for recoverin levels we have selected 4 groups with a negative "−" (0.03–0.09), enhanced "+" (0.1–1.7), highly increased

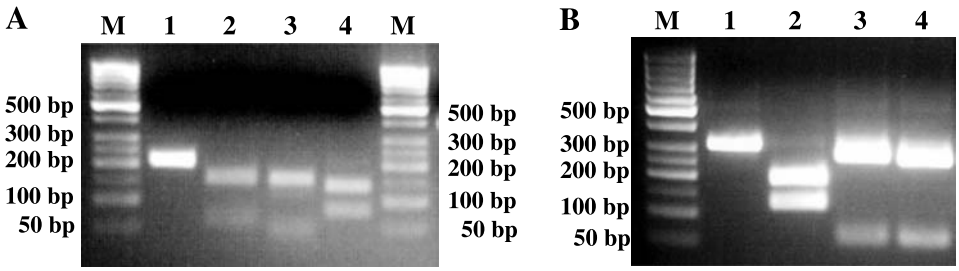

Fig. 2. Restriction analysis of the target RT-PCR products in 3% "wide range" agarose gel. M = 50 bp DNA ladder (GeneRuler, MBI Fermentas); lanes 1 = a non-digested amplification product; lanes 2, 3 and 4 = the amplification product digested with selected restriction endonucleases. Restriction analysis was performed using **A**). Alu I to get 58 and 151 bp fragments, Hae III to get 15, 44 and 150 bp fragments, Rsa I to get 77 and 132 bp fragments from the *209 bp β-actin* amplification product, **B**). Hpa II to get 121 and 188 bp fragments, Pst I to get 257 and 52 bp, Pvu II to get 256 and 53 bp fragments from the *309 bp recoverin* amplification product

Table 2. Quantification and classification of recoverin expression

Number of patient	C_t mean for β-actin	C_t mean recoverin	Relative expression of recoverin	Classification of recoverin expression
1	21.3	18.35	7.728	++
2	21.6	21.3	1.231	+
5	21.35	18.5	7.210	++
6	21.2	18.05	8.877	++
7	21.4	19.05	5.098	++
8	24.3	19.25	33.128	+++
11	21.15	19.25	3.732	++
13	21.45	19.9	2.928	++
15	17.1	20.7	0.082	−
16	15.35	20.6	0.026	−
17	17.65	21.5	0.069	−
18	18.45	21.1	0.159	+
19	17.55	21.05	0.088	−
20	17.55	21	0.092	−
23	16	20.9	0.033	−
24	15.6	19	0.095	−
25	16.6	19.3	0.154	+
26	16.3	19.7	0.095	−
27	14.75	18.8	0.060	−
28	16.05	18.9	0.139	+
29	24.55	23.85	1.625	+
30	25.5	23.35	4.438	++
31	22.35	24.05	0.308	+
32	24.9	22.7	4.595	++
33	25.5	22.95	5.856	++
34	24.65	23.95	1.625	+
36	17.2	20	0.144	+
37	18.5	20.7	0.218	+
38	16.25	19.3	0.121	+
40	16.2	19.4	0.109	+
41	21.65	21.3	1.275	+
43	24.25	21.85	5.278	++
44	24.55	23.4	2.219	++
46	16.4	19.55	0.113	+
50	18.8	18.85	0.966	+
51	22.8	19.4	10.556	+++
55	20.8	19	3.482	++
56	21.55	19.35	4.595	++

"++" (2.0–8.9) and extremely increased "+++" (10.0–33.0) expression. We found enhanced recoverin values in 63% and 70% of cases with proliferative and highly proliferative disease respectively, and with only two exceptions in all cases with no detectable proliferation (see Tables 4 and 5). The expression of recoverin was highly increased in 42%, 60%, and 41% of patients with proliferative, highly proliferative and no active proliferative disease. All smokers with one exception demonstrated enhanced recoverin values compared to only 68% of non-smokers. Highly increased recoverin expression was demonstrated in 62% and 28% of smokers and non-smokers respectively.

MMP-2 activities in serum

Based on relative values, MMP-2 activities were classified as negative "−" (0.–0.340), enhanced "+" (0.350–0.790), highly increased "++" (0.800–1.290) and extremely increased "+++" (higher than 1.300) (see Table 3). Enhanced MMP-2 activities were found in 72%, 67%, and 63% of patients with proliferative, highly proliferative and no active proliferative disease respectively (see Tables 4 and 5). The MMP-2 activities were highly increased in 44%, 44%, and 19% of patients with proliferative, highly proliferative and no active proliferative disease. 62% of smokers and 78% of non-smokers demonstrated enhanced MMP-2 activities; exactly one half of these subjects in each group (31% and 39% respectively) revealed highly increased MMP-2 serum levels.

MMP-9 activities in serum

For the evaluation of MMP-9 values (see Table 3) we classified negative "−" (0.–0.490), enhanced "+"

Table 3. Quantification and classification of the activity of MMP-2 and MMP-9

Number of patient	Activity of MMP-2 in relative units	Classification of MMP-2 expression	Activity of MMP-9 in relative units	Classification of MMP-9 expression
1	No data	No data	No data	No data
2	No data	No data	No data	No data
5	7.082	+++	1.109	++
6	0.245	−	1.257	++
7	0.224	−	0.619	+
8	0.082	−	1.528	+++
11	1.500	+++	1.370	++
13	0.221	−	0.744	+
15	0.894	++	0.837	+
16	0.499	+	0.138	−
17	0.519	+	0.744	+
18	0.488	+	0.180	−
19	0.421	+	0.956	++
20	1.249	++	1.364	++
23	1.294	++	1.416	++
24	0.262	−	1.020	++
25	0.286	−	0.644	+
26	0.778	+	2.090	+++
27	1.406	+++	1.521	+++
28	0.309	−	1.209	++
29	1.017	++	1.484	+++
30	0.755	+	0.863	+
31	1.004	++	1.350	++
32	1.431	+++	2.003	+++
33	0.317	−	0.867	+
34	0.893	++	0.785	+
36	0.350	+	1.376	++
37	2.181	+++	1.126	++
38	0.715	+	1.985	+++
40	0.569	+	0.721	+
41	0.954	++	0.801	+
43	0.551	+	0.879	+
44	1.285	++	0.896	+
46	0.500	+	0.325	−
50	0.765	+	0.490	−
51	0.335	−	0.273	−
55	0.287	−	0.262	−
56	0.451	+	0.589	+

(0.500–0.890), highly increased "++" (0.900–1.440) and extremely increased "+++" (higher than 1.490) activities (see Table 3). Enhanced MMP-9 activities were found in 72% and 81% of cases with and without proliferative disease respectively, and with only one exception, in all patients of the subgroup with highly proliferative disease (see Tables 4 and 5). The MMP-9 activities were highly increased in 56%, 67%, and 31% of patients with proliferative, highly proliferative and no active proliferative disease. In the subgroups B and C these numbers were 80%, 100%, 40% and 44%, 63%, 14% respectively (see Table 5). Almost an equal amount of smokers and non-smokers (85% and 83% respectively) demonstrated enhanced MMP-9 activities; 38% of smokers and 48% of non-smokers demonstrated highly activated MMP-9 serum levels.

Activation of recoverin, MMP-2 and MMP-9 in DM-patients

In DM-patients, two from three genes tested were highly activated in 28%, 22%, and 19% of cases with proliferative, highly proliferative and no active proliferative disease respectively (see Tables 4 and 5). All three genes were highly activated in 17% and 33% of patients with proliferative retinopathy and highly proliferative retinopathy respectively. In no one case without active proliferation we observed a simultaneously high activation of all

Table 4. Summarised information about the parameters evaluated

No. of Patient	Age	Sex	DM-Duration	Nicotine/ Years	Recoverin in leukocytes	MMP-2 activity in serum	MMP-9 activity in serum	Proliferation
Subgroup A with the DM-duration 3–9 years (10 people), 58.5 ± 8.2 years old								
34	59	M	3	+40	+	++	+	No data
56	65	M	3	+10	++	+	+	−
11	51	M	4	+8	++	+++	++	++
33	47	F	5	−	++	−	+	−
44	57	M	6	+40	++	++	+	+
16	68	M	6	−	−	+	−	+
17	66	M	7	−	−	+	+	+++
55	49	M	8	+40	++	−	−	++
37	54	F	8	−	+	+++	++	−
6	69	M	9	+40	++	−	++	−
Subgroup B with the DM-duration 10–19 years (11 people), 59.4 ± 5.9 years old								
38	56	M	10	+4	+	+	+++	−
24	63	M	12	−	−	−	++	+
20	65	M	12	+7	−	++	++	−
36	63	M	13	−	+	+	++	+
27	57	F	14	−	−	+++	+++	+
1	61	M	15	−	++	No data	No data	−
50	49	F	18	+35	+	+	−	−
41	53	M	18	−	+	++	+	−
46	67	F	18	−	+	+	−	+
26	54	F	19	−	−	+	+++	++
30	65	M	19	+18	++	+	+	−
Subgroup C with the DM-duration of DM more than 20 years (17 people), 60.7 ± 12.8 years old								
8	38	F	20	+5	+++	−	+++	+++
32	62	M	20	−	++	+++	+++	++++
31	65	F	20	−	+	++	++	+
5	67	F	20	−	++	+++	++	++
18	23	F	23	−	+	+	−	−
43	65	M	23	No data	++	+	+	−
15	67	M	23	−	−	++	+	+
7	63	M	25	−	++	−	+	+
25	67	M	27	−	+	−	+	−
29	67	M	29	−	+	++	+++	No data
2	65	M	30	−	+	No data	No data	++
13	66	M	31	+40	++	−	+	++
28	46	F	33	+1	+	−	+	−
23	66	F	33	−	−	++	++	++
40	70	M	38	−	+	+	+	−
19	66	F	39	−	−	+	++	−
51	70	F	43	−	+++	−	−	−

three genes tested, and from all subjects with the high proliferation only one patient demonstrated not even one highly increased marker.

Discussion

We performed a comparative *ex vivo* investigation of the differential expression of recoverin in mononuclear blood cells and both gelatinases A and B in serum as potential molecular markers for microvascular disease and vascular proliferation in the retina of DM-patients. Comparing three subgroups, selected on the basis of DM-duration, we did not observe any correlation between the DM-duration and the presence or severity of an active proliferative retinopathy, which is in agreement with recently published data by Cheung et al. (2001). To our surprise, there was also no positive correlation between individual HbA1c (long term blood glucose level) values and the status of proliferative retinopathy, which contradicts the literature (Lovestam-Adrian et al., 2001; Porta et al.,

Table 5. Evaluation of the correlation between the activity of both MMP-2 and MMP-9, expression of recoverin, smoking, and active proliferative retinopathy; the numbers are given in % from all patients in the DM-group and in subgroups A:B:C (see Table 1)

	All DM-patients	Patients with proliferation	Patients with high proliferation	Patients without proliferation	Smokers	Non-smokers
MMP-2	72	72	67	63	62	78
enhanced	40:90:56	80:80:63	67:100:67	50:100:57	67:100:0	75:83:75
MMP-9	83	72	**In all cases with 1 exception**	81	85	83
enhanced	80:80:88	60:80:100		100:80:71	83:75:100	75:83:83
Recoverin	76	63	70	**In all cases with 2 exception**	**In all cases with 1 exception**	68
enhanced	80:64:82	60:40:78	67:0:83			50:57:77
MMP-2 highly	36	**44**	**44**	**19**	31	39
increased	40:30:38	40:20:63	33:0:60	25:40:0	50:25:0	25:33:50
MMP-9 highly	44	**56**	**67**	**31**	38	48
increased	30:60:44	20:80:63	33:100:80	50:40:14	33:50:33	25:67:50
Recoverin highly	39	42	**60**	41	62	28
increased	60:18:41	60:0:56	67:0:67	75:33:29	83:25:67	25:14:31
Only one marker enhanced	**In 4 cases**	**In 3 cases**	**In 1 case**	**In 1 case**	**In 1 case**	**In 3 cases**
Two markers	47	**56**	**56**	**50**	46	43
enhanced	30:50:56	20:60:100	33:100:100	50:40:57	17:50:100	50:50:46
All three markers	42	33	33	44	46	39
enhanced	50:40:38	40:20:0	33:0:0	50:60:29	67:50:0	25:33:46
One marker	42	39	33	50	46	43
highly increased	40:50:25	20:60:33	33:100:20	50:60:43	50:50:33	25:67:38
Two markers	25	**28**	**22**	**19**	31	22
highly increased	30:20:25	20:20:33	0:0:40	50:20:0	33:25:33	25:17:23
All three markers	8	**17**	**33**	**0**	8	9
highly increased	10:0:13	20:0:22	33:0:40	**0:0:0**	17:0:0	0:0:15
No one marker	22	**18**	**0**	**31**	15	26
highly increased	20:30:37	40:20:12	**with 1 exception**	0:20:57	0:25:33	50:16:24

2001; Henricsson et al., 2003), but could be explained by the fact that in our study we had only one time-point to look at. However, our observation indicates that the prediction of the occurrence or the severity of diabetic retinopathy based on a single HbA1c value alone could be extremely difficult.

The hybridisation to Atlas Array revealed an increased expression of recoverin in mononuclear blood cells of DM-patients compared to non-diabetic controls. The recoverin gene mapped to human chromosome 17 (Murakami et al., 1992) encodes a photoreceptor protein that became intensively studied due to its implication in cancer associated retinopathy (CAR), an ocular manifestation of a paraneoplastic syndrome that is clinically characterised by progressive visual impairment similar to retinitis pigmentosa (Thirkill et al., 1993). As a possible cause of the retinal degeneration seen in CAR an aberrant expression of recoverin with the concomitant production of anti-recoverin antibody has been proposed (Ohguro and Nakazawa, 2002). Anti-recoverin antibodies have been suggested to reach the retina via the peripheral circulation and to block recoverin function such as the inhibition of Ca^{2+}-dependent rhodopsin phosphorylation, thus inducing retinal apoptosis. A possible involvement of recoverin, expressed in mononuclear blood cells, in the pathomechanism of diabetic retinopathy has not been investigated yet. Our measurements demonstrated the high expressional levels of recoverin in 39% of all DM-patients tested and in 60% of the subgroup with highly proliferative retinopathy in contrast to only 41% of cases with no active proliferation. These results indicate an association of high recoverin expression in mononuclear blood cells with neovascularisation in diabetic retinopathy. The mechanism by which the enhanced recoverin expression in circulating leukocytes might be involved in the pathomechanism of diabetic retinopathy needs to be further clarified. Noteworthy, almost all smokers also demonstrated increased recoverin values compared to only 68% of non-smokers. In addition, more than twice as many smokers had highly increased recoverin levels compared to non-smokers. An interpretation of this result could be only speculative at the moment and is open for further investigations.

An important role of both MMP-2 and MMP-9 has been proposed in the pathomechanism of diabetic vasculopathy (Uemura et al., 2001; Mene et al., 2001). Indeed, our measurements of the activity in serum showed an increased or even highly increased level of both MMP-2 and MMP-9 in the majority of DM-patients compared to non-diabetic controls. Particularly high levels were observed for MMP-9 activity, and those were increased in patients with active proliferative disease. With only one exception noted, the MMP-9 activity was increased in all patients with highly active proliferative retinopathy. This correlation can be seen well in subgroups B and C with a longer DM-duration of 10–19 and 20–43 years respectively. These findings indicate a specific role of MMP-9 in the molecular mechanisms of active diabetic proliferation. Highly increased activities of both MMP-2 and MMP-9 were observed in more than twice as many cases with highly active proliferative retinopathy compared to patients with no detectable active proliferation. The increased activity of both MMPs in serum of DM-patients appears to play a key role in tissue remodelling as a part of retinal neovascularisation.

An interesting aspect that warrants further investigation is the effect of ocular anti-proliferative therapy such as retinal laser coagulation or cryocoagulation on a possible activation of the molecular markers tested in this study. Coagulation of retinal tissue leads to a reduction of proliferative activity and thus might also exert a specific effect on the investigated genes. In addition, future studies should be aimed at comparing proliferative vasculopathy due to DM and other diseases.

From our data we conclude that DM-patients with active retinal vascular proliferation demonstrated a high activation of all three genes investigated compared to patients with no active retinopathy. While the former patients always demonstrated at least one highly up-regulated gene out of the three genes tested, the latter patients without detectable proliferation did not demonstrate a high activation of all three genes simultaneously. These findings allow us to propose the *ex vivo* quantification of gelatinase activity in serum and expression of recoverin in mononuclear blood cells as a molecular imaging system for the prediction of active proliferative retinopathy in DM-patients. Possibly, with establishing a time course of specific molecular events, these patients might be monitored more effectively for the initiation of appropriate therapy.

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