INTERFERENCE OF PERITONEAL DIALYSIS FLUIDS WITH CELL CYCLE MECHANISMS

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♦ **Introduction:** Peritoneal dialysis fluids (PDF) differ with respect to osmotic and buffer compound, and pH and glucose degradation products (GDP) content. The impact on peritoneal membrane integrity is still insufficiently described. We assessed global genomic effects of PDF in primary human peritoneal mesothelial cells (PMC) by whole genome analyses, quantitative real-time polymerase chain reaction (RT-PCR) and functional measurements.

♦ **Methods:** PMC isolated from omentum of non-uremic patients were incubated with conventional single chamber PDF (CPDF), lactate- (LPDF), bicarbonate- (BPDF) and bicarbonate/lactate-buffered double-chamber PDF (BLPDF), icodextrin (IPDF) and amino acid PDF (APDF), diluted 1:1 with medium. Affymetrix GeneChip U133Plus2.0 (Affymetrix, CA, USA) and quantitative RT-PCR were applied; cell viability was assessed by proliferation assays.

♦ **Results:** The number of differentially expressed genes compared to medium was 464 with APDF, 208 with CPDF, 169 with IPDF, 71 with LPDF, 45 with BPDF and 42 with BLPDF. Out of these genes 74%, 73%, 79%, 72%, 47% and 57% were downregulated. Gene Ontology (GO) term annotations mainly revealed associations with cell cycle ($p = 10^{-35}$), cell division, mitosis, and DNA replication. One hundred and eighteen out of 249 probe sets detecting genes involved in cell cycle/division were suppressed, with APDF-treated PMC being affected the most regarding absolute number and degree, followed by CPDF and IPDF. Bicarbonate-containing PDF and

BLPDF-treated PMC were affected the least. Quantitative RT-PCR measurements confirmed microarray findings for key cell cycle genes (CDK1/CCNB1/CCNE2/AURKA/KIF11/KIF14). Suppression was lowest for BPDF and BLPDF, they upregulated CCNE2 and SMC4. All PDF upregulated 3 out of 4 assessed cell cycle repressors (p53/BAX/p21). Cell viability scores confirmed gene expression results, being 79% of medium for LPDF, 101% for BLPDF, 51% for CPDF and 23% for IPDF. Amino acid-containing PDF (84%) incubated cells were as viable as BPDF (86%).

♦ **Conclusion:** In conclusion, PD solutions substantially differ with regard to their gene regulating profile and impact on vital functions of PMC, i.e. on cells known to be essential for peritoneal membrane homeostasis.

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In the past decades, peritoneal dialysis (PD) has been established as an effective and economic alternative to hemodialysis (HD) for the treatment of end stage renal disease. The long-term success of PD, however, is still limited by progressive deterioration of peritoneal morphology and transport functions (1). Prolonged exposure of the peritoneum to non-physiological PD fluids (PDF) is associated with progressive submesothelial fibrosis, vasculopathy, neoangiogenesis, and loss of the mesothelial cell layer (2). Nearly 50% of patients must switch to hemodialysis within 4 to 5 years of treatment

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due to degenerative changes of the peritoneal tissue that ultimately lead to ultrafiltration failure (3,4). The poor biocompatibility of conventional PDF is based on their high glucose and lactate concentrations, the acidic pH, and numerous toxic glucose degradation products (GDP) (5–8). The latter have proven toxic by impairing mesothelial cell proliferation and repair mechanisms (9–12), and by inducing vascular endothelial growth factor (VEGF) in cultured rat mesothelial and human endothelial cells (13). Glucose, itself, impairs numerous cellular functions, and this is enhanced by the use of lactate as the buffering substance at an acidic pH (14). High concentrations of glucose activate protein kinase C, induce reactive oxygen species and upregulate the expression of transforming growth factor β 1 and fibronectin in human peritoneal mesothelial cells (PMC) (15,16).

Different PD fluids with an improved biocompatibility profile have been introduced. Glucose degradation products have been reduced by the separation of glucose at a very low pH from the buffer during sterilization and storage. Glucose-based PDF can be replaced at least in part by icodextrin and amino acid-containing

solutions, the lactate buffer, which is particularly toxic at a low pH, and by PDF containing bicarbonate at a normal to physiological pH (Table 1)(17). Numerous *in vitro*, *ex vivo* and clinical studies support the notion of improved biocompatibility (10,23–28). The clinical impact, however, is still debated (29). Peritoneal mesothelial cells play an essential role in maintaining peritoneal membrane homeostasis and thus structural and functional integrity. They secrete numerous cytokines and growth factors (30–32), contribute to peritoneal host defense (33) and prevent local frictions and adhesions by secretion of active surface substances and lubricants such as cancer antigen (CA) 125. CA125 has been used as a PD effluent surrogate marker of PMC mass (34). Effluent CA125 concentrations decline with conventional but not with low GDP solutions (10,26), suggesting major differences in PMC mass and viability in PD patients treated with different PDF. The precise fate of the PMC, however, remains unclear. *In vivo* exposure of PMC to high glucose PDF accelerates PMC senescence and elimination via the dialysate (35). Other PMC eventually undergo epithelial to mesenchymal-transition (EMT) in

TABLE 1
Composition of PDF and GDP Content (17–22)

	BPDF	BLPDF	LPDF	CPDF	IPDF	APDF
Electrolytes (mmol/L)						
Na+	134	132	134	134	133	132
K+	—	—	—	—	—	—
Cl-	104.5	95	101.5	103.5	96	105
Ca ²⁺	1.75	1.25	1.75	1.75	1.75	1.25
Mg ²⁺	0.5	0.25	0.5	0.5	0.25	0.25
Buffer						
Lactate	—	15	35	35	40	40
HCO ₃ ⁻	34	25	—	—	—	—
Osmotic agents (g/L)						
glucose	22.73	22,7	22.73	22.73	—	—
icodextrin	—	—	—	—	75	—
amino acids	—	—	—	—	—	11
pH	~7,4	~7,0	~7,0	~5,5	5 - 6	~6,7
Osmolarity (mmol/L)	399	395	401	401	301	365
GDP contents (μmol/L)						
total GDP	28,5–45,2	163,4–228	24–32,5	273–335	46,2–63,8	3,8
3,4-DGE	0,2–2,7	11–14,3	<0,4–5,3	4,9–16,2	<0,4–3,0	n.d.

PDF = peritoneal dialysis fluids; GDP = glucose degradation products; BPDF = bicarbonate-buffered PDF; BLPDF = bicarbonate/lactate-buffered PDF; LPDF = lactate-buffered PDF; CPDF = conventional PDF; IPDF = icodextrin-containing PDF; APDF = amino acid-containing PDF; n.d. = not determined.

response to PDF-associated stress and may contribute to peritoneal membrane deterioration (36). To assess the global effects of different PD fluids on PMC function and fate we conducted whole genome microarray analyses, followed by a quantitative RT-PCR approach, as well as functional measurements.

MATERIALS AND METHODS

HUMAN PERITONEAL CELL ISOLATION AND CELL CULTURE

Human PMC were isolated from specimens of omentum obtained from consenting, non-uremic patients undergoing elective abdominal surgery due to diseases not involving the omentum. Approval was obtained from the local ethical committee; written informed consent was obtained from each patient. Cells were isolated and characterized as described elsewhere (37). PMC were propagated in the M199 culture medium (Biochrom AG, Berlin, Germany), supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 0.4 µg/mL hydrocortisone, 0.5 µg/mL insulin, 0.5 µg/mL transferrin and 10% fetal calf serum (FCS). Cells were maintained at 37°C in humidified 5% CO₂. Purity of the mesothelial cells was validated by the uniform cobblestone appearance at confluence and immunofluorescent staining with mesothelial markers (Cytokeratins 8 and 18, Vimentin) without staining of von Willebrand factor (vWF). Ribonucleic acid (RNA) isolation was performed with cells in the first to third passages. Peritoneal mesothelial cells were incubated with different PD solutions for 24 hours, diluted 1:1 with media: conventional peritoneal dialysis fluid (CPDF; CAPD 2,3%, Fresenius Medical Care, Bad Homburg, Germany), lactate-buffered, neutral pH peritoneal dialysis fluid (LPDF; balance 2,3%; Fresenius Medical Care, Bad Homburg, Germany), bicarbonate-buffered, neutral pH dialysis fluid (BPDF; bicaVera 2,3%; Fresenius Medical Care, Bad Homburg, Germany), bicarbonate/lactate-buffered, neutral pH peritoneal dialysis fluid (BLPDF; Physioneal; Baxter Healthcare Corporation, Deerfield, IL, USA), icodextrin-containing peritoneal dialysis fluid (IPDF; Extraneal; Baxter Healthcare Corporation, Deerfield, IL, USA), and amino acid-containing peritoneal dialysis fluid (APDF; Nutrineal; Baxter Healthcare Corporation, Deerfield, IL, USA). In a further set of experiments PMC were incubated with increasing concentrations of 3-DG (Sigma-Aldrich, Munich, Germany) and 3,4-DGE (LC Scientific Inc., Concord, Canada), respectively, for 24 h. Cytotoxicity was assessed by determination of supernatant LDH concentrations.

RNA EXTRACTION AND PROCESSING

For RNA isolation, cells were plated at a density of 2.5×10^5 cells/well in six-well plates. Ribonucleic acid was isolated using TRI Reagent (Sigma-Aldrich, Munich, Germany) according to the manufacturer's directions, checked for integrity on an agarose gel and quantified photometrically.

WHOLE-GENOME RNA MICROARRAY ANALYSIS

An RNA microarray analysis was carried out on RNA isolated from human PMC from 4 different donors using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, CA, USA) as described in the Affymetrix GeneChip 3" IVT Express Kit User Manual.

Hybridization, washing and staining of the array was done on a GeneChip Fluidics Station 450 according to the standard Affymetrix GeneChip protocol (Version 2). Arrays were scanned on the Affymetrix GeneChip Scanner 3000 with G7 update.

DATA ANALYSIS

Affymetrix raw data (CEL files) were processed using the Genedata Expressionist Refiner Array software (Version 6.1; Genedata, Basel, CH). Quality classification applying default parameters produced the following results: 34 chips were flagged as GOOD (94.4%), 1 chip was flagged as MEDIUM (2.8%) due to slightly sub-optimal control gene statistics (5" to 3" ratio), and 1 chip was flagged as BAD (2.8%) due to an above-average proportion of masked features (1.4%). All chips were included in further analysis since there were no indications of broad-scale changes in intensity values caused by artefacts in the 2 chips labeled as MEDIUM or BAD, respectively. Raw data have been condensed using the GeneChip Robust Multi-array Averaging (GC-RMA) algorithm (38).

PRINCIPLE COMPONENT ANALYSIS

Prior to performing additional statistical analyses, the data set was pre-processed by principle component analysis (PCA) in order to assure an unbiased approach to the data. The main application of PCA is to reduce the number of variables, as well as to detect structure in the relationship between variables and to classify these. Two or more of these variables are expressed by a single factor termed principle component, which describes the variance of the data, allowing for the evaluation of their consistency. For this purpose, all 54,675

ProbeSets present on the Affymetrix GeneChip have been used and no pre-filtering has been applied. Principle component analysis was visualized in a 3-dimensional matrix, showing the variance of samples and their respective treatment.

BIOLOGICAL FUNCTIONS AND PATHWAY ANALYSIS

In the following, condensed data were analyzed using the Genedata Analyst (Version 6.1; Genedata, Basel, CH). A multiple-factor analysis of variance (ANOVA) was used to identify genes significantly changed between treatment groups. ProbeSets with a permutation Q-value below 0.001 (calculated from 50 repeats of balanced permutations of group assignments) and a fold-change larger than 2 were selected for further analysis and were used for *k*-means clustering (*k* = 2) (*k* = 4 for supplementary Figure S1). For visibility reasons, expression profiles were re-scaled to a common mean value and displayed on a logarithmic scale of arbitrary values. Supplementary Table ST1 provides a comprehensive list of genes found to be significantly regulated after ANOVA as well as after individual *t*-tests comparing a treatment to medium control. For further statistics, Fisher's exact test using Gene Ontology (GO) term annotation was performed on clustered groups after mapping ProbeSets onto gene symbols to avoid bias due to genes represented by multiple ProbeSets.

QUANTITATIVE REAL-TIME PCR VALIDATION

To validate differentially expressed genes identified by microarray analysis, quantitative real-time polymerase chain reaction (RT-PCR) was performed on the same set of RNA that was used for the microarray experiments.

Two micrograms of total RNA were reverse transcribed in 20 μ l volume with random primers using the High Capacity cDNA Reverse Transcription Kit (Part No. 4368814, Applied Biosystems, CA, USA) as recommended. Reverse transcription reactions were diluted to 100 μ l and stored at -80°C.

Quantitative RT-PCR was performed with the ABI Prism 7900HT Sequence Detector (Applied Biosystems, CA, USA) using the TaqMan Universal PCR Master Mix protocol and TaqMan assays as recommended by the manufacturer (Applied Biosystems, CA, USA). All reactions were carried out in a 20 μ l final volume containing 10 μ l Master Mix, 1 μ l of the specific assay and 9 μ l diluted cDNA corresponding to 9–18 ng of total RNA. The amplification was performed under the following conditions: 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for

1 min. Target gene expression was normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous reference by computing the difference between the respective cycle threshold (Δ Ct). Differential expression was calculated by the $\Delta\Delta$ Ct method. All PCR reactions were performed in quadruplicate.

CELL PROLIFERATION ASSAY

Peritoneal mesothelial cells were plated in quadruplicate wells in a 100 mL volume per well of a 96-well microtiter plate. The cells were grown to 70% confluence (48 h) followed by 24 hours incubation in starvation medium (no FCS). For treatment, cells were incubated for 24 hours with the different PDF mixed 1:1 with starvation medium. Medium only, mixed with different PDF served as negative control. Cell viability was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Optical densities of the samples were measured at 490 nm using an automatic microplate reader. Values from treated cells were compared with values generated from the negative control and reported as percent viability. Significance was assessed by unpaired *t*-tests for values referring to APDF; all others values were determined by one-way ANOVA.

CASPASE-3-ASSAY

Human PMC were incubated with different PDF for 24 hours and lysed. Caspase-3-activity was detected and quantified by ELISA (R&D Systems Inc., Minneapolis, USA) after modifying the active Caspase-3 large subunit with biotin-ZVKD-fmk following the manufacturer's instructions.

RESULTS

PRINCIPLE COMPONENT ANALYSIS

Principle component analysis was applied as a means of condensing the microarray data, thereby being able to identify possible patterns or highlighting differences or similarities between data sets, respectively. It revealed a clear separation of PMC samples treated with APDF from those treated with medium. Bicarbonate containing PDF and BLPDF-treated PMC grouped along the principle components 2 and 3. Peritoneal mesothelial cells incubated with LPDF, IPDF and CPDF were located in between. Peritoneal mesothelial cells gene expression profiles from different donors were separated along

principle component 1. Thus, treatment effects were superior as compared to potential donor related effects (Figure 1).

MICROARRAY ANALYSIS AND CLUSTERING

Microarray analyses of PMC identified two clusters of genes. The expression profile of Cluster 2 indicated a general upregulation of genes, with CPDF, IPDF and APDF treatments showing the strongest increases compared to control medium. Cluster 1 showed a downregulation with all treatments, expressed the strongest in APDF-treated cells. Genes from BPDF- and BLPDF-treated cells were least affected in both clusters. In general, different treatments showed large differences in the magnitude of expression changes (Figure 2, see also supplementary Figure S1).

Figure 1 — Principle component analysis (PCA) of GeneChip Robust Multi-array Averaging (GC-RMA) condensed data. Experiments are colored according to treatment with different PDF and shapes according to donor. Principle component analysis reveals a clear separation of PMC samples treated with APDF from those treated with medium. BPDF- and BLPDF-treated PMC group along the principle components 2 and 3. Peritoneal mesothelial cells incubated with LPDF, IPDF and CPDF are located in between. Peritoneal mesothelial cells gene expression profiles from different donors are separated along principle component 1. Shapes: square = donor 1; circle = donor 2; cross = donor 3; staggered square = donor 4. Colors: light blue = control; green = BPDF; yellow-green = BLPDF; red = LPDF; dark blue = CPDF; orange = IPDF; purple = APDF. PMC = peritoneal mesothelial cells; PDF = peritoneal dialysis fluids; BPDF = bicarbonate-buffered PDF; BLPDF = bicarbonate/lactate-buffered PDF; LPDF = lactate-buffered PDF; CPDF = conventional PDF; IPDF = icodextrin-containing PDF; APDF = amino acid-containing PDF.

The number of differentially expressed genes compared to the control medium, detected via specific ProbeSets, differed substantially between double chamber PDF and APDF, with 10-fold more genes passing the threshold of significance of $p < 0.001$ with the latter. Single chamber PDF and IPDF induced moderate gene deregulation (Table 2). It is noteworthy that 75% of genes were down-regulated with CPDF, IPDF and APDF.

Generally, genes found to be up- or down-regulated significantly in one of the treatments show the tendency to be regulated in the same direction, though not always significantly, with the other treatments, meaning that differences between treatments are of quantitative rather than qualitative nature.

GENE ONTOLOGY TERM ANNOTATIONS OF DIFFERENTIALLY REGULATED GENES

To classify the function of genes found by ANOVA to be regulated in comparison to control medium, a GO term annotation was performed. In the group of suppressed genes, associations with the cell cycle were highly enriched as compared to the gene universe represented on the chip. Sixteen out of the top 20 GO terms displayed in Figure 3a are essentially attributed to cell cycle processes. The remaining 4 terms (phosphatidylinositol-mediated treatment, microtubule-based movement, DNA-repair and nucleotide-excision repair) are also implicated in cell cycle processes. Highest associations among the suppressed genes were identified for the GO terms cell cycle, cell division, mitosis, and DNA replication (Figure 3a). Lower, but still significant associations were detected for GO terms of upregulated genes. They belong to various categories such as metabolic/catabolic processes (Figure 3b).

GENE EXPRESSION PROFILES OF K-MEANS CLUSTERED GENES

One hundred and ninety-six genes – represented by 249 ProbeSets – found to be suppressed by treatment were annotated with interaction data from ConsensusPathDB (available at: <http://cpdb.molgen.mpg.de/>). The largest network found contained 42 genes, in this particular case from APDF-treated cells, which demonstrated their functional relationship within cell cycle processes (Figure 4).

A heatmap of cell cycle genes found to be suppressed in treated samples is represented in Figure 5a. One hundred and eighteen out of a total of 249 ProbeSets detecting gene suppression are known to be involved in cell cycle or cell division. The APDF-treated samples followed by CPDF and IPDF were downregulated the most, both regarding

Figure 2 — K-means clustering of ProbeSets identified by ANOVA. The cluster of genes showing increased expression after treatment of PMC with different PDF compared to medium control is displayed in blue. The cluster of genes showing decreased expression with all treatments compared to medium control is displayed in red. The least regulation compared to control is seen for BPDF and BLPDF, the highest for APDF. PMC = peritoneal mesothelial cells; PDF = peritoneal dialysis fluids; BPDF = bicarbonate-buffered PDF; BLPDF = bicarbonate/lactate-buffered PDF; LPDF = lactate-buffered PDF; CPDF = conventional PDF; IPDF = icodextrin-containing PDF; APDF = amino acid-containing PDF.

TABLE 2
Number of Genes Regulated in Human PMC Treated
with Different PDF as Compared to Medium Control^a

	Suppressed	Number of genes Induced	Total
BPDF	21	24	45
BLPDF	24	18	42
LPDF	51	20	71
IPDF	133	36	169
CPDF	151	56	208
APDF	343	121	464

PMC = peritoneal mesothelial cells; PDF = peritoneal dialysis fluids; BPDF = bicarbonate-buffered PDF; BLPDF = bicarbonate/lactate-buffered PDF; LPDF = lactate-buffered PDF; IPDF = icodextrin-containing PDF; CPDF = conventional PDF; APDF = amino acid-containing PDF.

^a applying a *p*-value of *p* < 0.001.

the absolute number and the degree of regulation (Figure 5a). In contrast, gene expression was least affected by BPDF and BLPDF treatment. Figure 5b shows a heatmap

of all upregulated genes. Analogous to Figure 5a, genes were upregulated the least with BPDF and BLPDF treatment, whereas they show a marked upregulation with IPDF and APDF.

QUANTITATIVE REAL-TIME PCR VALIDATION

Quantitative RT-PCR measurements confirmed micorarray findings for key genes mediating the four principle phases of the cell cycle. Essential genes for passing G1/S or G2/M checkpoint control and genes regulating M phase were suppressed by PDF (Figure 6). Suppression of these genes was lower for BPDF and BLPDF (Figure 6A and B), and some genes (CDK2, CCNB1, CCNE2, KIF11 and SMC4 for BPDF; CDK2 and SMC4 for BLPDF) were upregulated. All PDF upregulated 3 out of 4 cell cycle repressors.

CELL PROLIFERATION CAPACITY

Cell proliferation scores partially confirmed the trend seen in gene expression results. Viability was the highest for BLPDF (101% of medium). Contrasting the previous findings, APDF-incubated cells were as viable

Figure 3a — Fisher's exact test: GO term annotations of suppressed genes in PMC following treatment with different PDF. GO = gene ontology; PMC = peritoneal mesothelial cells; PDF = peritoneal dialysis fluids.

Figure 3b — Fisher's exact test: GO term annotations of induced genes in PMC following treatment with different PDF. GO = gene ontology; PMC = peritoneal mesothelial cells; PDF = peritoneal dialysis fluids.

as BPDF (84%). The viability score for LPDF-treated cells was 79% and 51% for CPDF, respectively. Icodextrin-incubated cells displayed the lowest viability score (23%) (Figure 7). Caspase 3 activity increased with BPDF (136.7 ± 7.8) and CPDF ($125.4 \pm 8.8\%$ vs medium control; both $p < 0.05$) and tended to increase with BLPDF (119.6 ± 23.4), LPDF (131.8 ± 19.8) and IPDF (39.4 ± 23) but not with APDF ($83 \pm 27,1\%$ of medium control; all

$p = ns$). To further delineate the impact of the PD fluids on cell proliferation, we measured the effect of the GDP 3-DG and 3,4-DGE on five different cell cycle genes. Incubation of PMC with 3-DG for 24 h had no effect on AURKA, CDK1, CCNE2, CCNB1 and KIF14 expression at concentrations up 500 $\mu\text{mol/L}$ and did not result in any significant increase of medium LDH concentrations. In contrast, 3,4-DGE downregulated 4 out of the 5 genes,

Figure 4 — Interaction network of suppressed genes using APDF-treated PMC as an example. The functional relationship within cell cycle processes is demonstrated by 42 genes contained in this single network. Genes are colored according to their median expression relative to control (red = 1; blue < 0.2). APDF = amino acid-containing peritoneal dialysis fluid; PMC = peritoneal mesothelial cells.

while CCNE2 was upregulated (Figure 8). A significant 58 to 152% increase in medium LDH was observed at 3,4-DGE concentrations of 5-100 $\mu\text{mol/L}$.

DISCUSSION

For the first time, the present study assessed the effect of different, currently available PDF on PMC at the whole-genome level, to expand the current understanding of peritoneal membrane alterations that are ultimately associated with PDF exposure. Bicarbonate PDF treatment

resulted in little gene (dys)-regulation in PMC, followed by lactate-based, low GDP solution. High levels of gene regulation occurred with conventional and icodextrin PDF and a very high level with amino acid-based fluid. The vast majority of the genes were directly or indirectly involved in cell cycle processes. These findings were reproduced by quantitative RT-PCR and, except for amino acid-based fluids, also in cell proliferation assays. Our findings underline the role of GDPs and of the osmotic agent in PMC viability, whereas the buffer compound at a neutral pH seems to be of less importance.

Figure 5 — Heatmaps of deregulated genes in PMC treated with different PDF. An overview of all genes analyzed is shown at the left of each figure; genes showing the largest overall effect (bordered area from heatmap on the left) are represented in the main part of the figures. Expression values are color-coded relative to medium control as given in the color scale on the right. Brighter green colour indicates stronger gene downregulation (Figure 5a), brighter red colour stronger upregulation (Figure 5b). PMC = peritoneal mesothelial cells; PDF = peritoneal dialysis fluids; CDC2 = alias for CDK1; BPDF = bicarbonate-buffered PDF; BLPDF = bicarbonate/lactate-buffered PDF; LPDF = lactate-buffered PDF; CPDF = conventional PDF; IPDF = icodextrin-containing PDF; APDF = amino acid-containing PDF.

Figure 6 — Deregulated cyclins and cyclin-dependent kinases (CDKs) (dark gray bars), mitosis-related genes (white bars), and cell cycle repressors/inhibitors (light gray bars) in PMC following treatment with different PDF relative to control medium as determined by RT-PCR. PMC = peritoneal mesothelial cells; PDF = peritoneal dialysis fluids; RT-PCR = real-time polymerase chain reaction; BPDF = bicarbonate-buffered PDF; BLPDF = bicarbonate/lactate-buffered PDF; LPDF = lactate-buffered PDF; CPDF = conventional PDF; IPDF = icodextrin-containing PDF; APDF = amino acid-containing PDF; CDK1 = cyclin-dependent kinase 1; CDK2 = cyclin-dependent kinase 2; CCNB1 = cyclin B1; CCNE2 = cyclin E2; AURKA = aurora kinase A; KIF11 = kinesin family member 11; KIF14 = kinesin family member 14; SMC4 = structural maintenance of chromosomes 4; TP53 = tumor protein p53; BAX = BCL2-associated X protein; CDKN1A = cyclin-dependent kinase inhibitor 1A (p21); CDKN3 = cyclin-dependent kinase inhibitor 3.

Figure 7 — Cell viability of PMC treated with different PDF assessed by proliferation assay. Viability scores (in % of control) are: 101 for BLPDF, 86 for BPDF, 84 for APDF, 79 for LPDF, 51 for CPDF and 23 for IPDF. Different superscripts indicate significant differences between groups ($p < 0.05$). PMC = peritoneal mesothelial cells; PDF = peritoneal dialysis fluids; BPDF = bicarbonate-buffered PDF; BLPDF = bicarbonate/lactate-buffered PDF; LPDF = lactate-buffered PDF; CPDF = conventional PDF; IPDF = icodextrin-containing PDF; APDF = amino acid-containing PDF.

Peritoneal mesothelial cells are particularly suited for analyses of PDF-related effects on the peritoneal membrane since they are primarily exposed to PDF and have a plethora of functions essential for peritoneal membrane homeostasis and thus morphological and functional integrity. PMC secrete cytokines involved in the local immune defense system, in inflammatory, fibrotic and neoangiogenic processes and secrete lubricants such as CA125 to prevent local frictions and adhesions (30–34,39). We now demonstrate that, depending on the GDP content, osmotic agent and buffer composition, currently available PDF have profound effects on the PMC genome regulation and on cell viability. In line with these findings, clinical studies demonstrated marked differences in effluent CA125 concentrations, a marker for viable PMC mass. Low GDP fluids 2- to 3-fold increase effluent CA125 concentrations as compared to conventional PD solutions (10,26). *Ex vivo*, effluent PMC growth rates are only slightly higher with IPDF than with conventional glucose PDF (40). *In vitro*, prolonged exposure to

Figure 8 — Regulation of cell cycle genes by 3,4-DGE. Peritoneal mesothelial cells were incubated with increasing concentrations of 3,4-DGE (x-axis) and gene expression quantified by RT-PCR. Changes relative to control medium are given (y-axis). CDK1 = cyclin-dependent kinase 1; CCNB1 = cyclin B1; CCNE2 = cyclin E2; AURKA = aurora kinase A; KIF14 = kinesin.

high GDP solutions reduces PMC proliferation, increases apoptosis rate and induces senescence (41,42).

The PCA of our microarray data demonstrated that treatment-related effects were superior to potential donor-related effects. Cluster and specific ProbeSets analyses revealed large differences in the magnitude of gene expression changes depending on the PDF treatment. According to the GO term annotations, the majority of the highly regulated genes are involved in cell cycle processes. In terms of number of genes and degree of downregulation, the changes were most pronounced in APDF treated PMC, followed by CPDF and IPDF, while gene expression was least affected by BLPDF and BPDF treatment.

To further substantiate these findings, RNA levels of key molecules of the cell cycle such as cyclins and cyclin-dependent kinases (CDKs), mitosis-related genes as well as cell cycle repressors/inhibitors, were checked using quantitative RT-PCR measurements. Cyclins are the primary modulators of CDKs and regulate the temporal coordination of each cell cycle event through their distinct expression and degradation pattern. Binding to their specific CDK partner promotes the progression into the next cell cycle phase. Cyclin-dependent kinase 1 and its partner cyclin B1 (CCNB1) are active subunits of the M-phase promoting factor and are essential for the G2/M phase transition (43); CDK2 and its cyclin E2 (CCNE2) coordinate the transition from G1 to S-phase. The observed downregulation of PMC CDK1, CCNB1, and CCNE2 by IPDF, APDF, and CPDF indicates a halt in cell cycle progression. CDK2, involved in G1/S transition, progression through S-phase, activation of DNA damage response (44) was upregulated by all PDF except LPDF.

In addition, we analyzed the regulation of key repressors/inhibitors of the cell cycle. p21 (CDKN1A), a CDK2/cyclin E inhibitor essential for arresting the cell cycle at the G1/S checkpoint (45), and p53, which orchestrates a network of signaling pathways essential for stress-induced cell growth and apoptosis (46), were both upregulated by PDF. p53 induces the expression of p21 (47) and of BAX and thus apoptosis (48). In line with this, activity of caspase 3, a key enzyme of apoptosis, was upregulated by the PDF. In addition, p53/p21 transcriptionally repress CDK1 (49), further controlling the G2/M checkpoint transition and cell cycle progression. In summary, the downregulation of essential genes promoting the progression through the cell cycle together with the upregulation of cell cycle repressors point towards a slowdown of the replication and division of PMC, ascendingly clear-cut for CPDF, IPDF and APDF.

Genes of the kinesin family are critical for mitosis. KIF11 encodes for a motor protein that is involved in proper spindle formation (50). Silencing of KIF14 disturbs chromosome congression and alignment, resulting in a prolonged delay at the metaphase-to-anaphase transition (51). The temporally and spatially controlled expression and activation of the Aurora A kinase (AURKA) is inevitable for centrosome maturation, entry into mitosis, spindle formation and function as well as cytokinesis (52). All of these tested genes were downregulated under CPDF, IPDF and APDF, again suggesting a delay in progression in yet another phase of the cell cycle. On the other hand, the genes encoding proteins essential for the mitosis phase of the cell cycle were little or unaffected when PMC were treated with BPDF and BLPDF. The gene encoding a protein conducting chromosome condensation as a prerequisite for chromosome segregation during mitosis, structural maintenance of chromosomes 4 (SMC4) (53), was even upregulated.

Exposure to increasing concentrations of 3-DG up to 500 $\mu\text{mol/l}$ did neither result in gene regulation nor in toxicity. This is in line with previous findings for several GDPs (54,55). In contrast, 3,4-DGE, a highly reactive GDP in PDF (56), downregulated 4 out of the 5 cell cycle genes studied at concentrations as present in PDF. They are involved in late phases of the cell cycle, whereas the upregulated gene, CCNE2, is involved in the G1/S phase. Thus, our findings indicate that part of the effects of PDF on the mesothelial cell cycle are exerted via specific GDP.

Consistent with the microarray and quantitative RT-PCR analyses, proliferation capacity of PMC were least affected with BLPDF, BPDF and LPDF treatment. Icodextrin, despite lower GDP concentrations, had a more pronounced effect on cell proliferation than CPDF, indicating that other factors than GDP are involved. APDF regulated numerous genes that are involved in cell cycle processes. The PMC proliferation assay, however, did not reveal a respective impairment. This is in contrast to the effect of both conventional and icodextrin PDF and may be related to an additional nutritional effect of the amino acids (57) in our cell model. Amino acids in PDF do not only allow for phosphate-free amino acid supply and serve as osmotic agent, but transmit information by binding to receptors (58,59), activating specific intracellular signaling pathways and regulating gene groups (60,61). The biocompatibility of amino PDF is debated. Whereas some studies suggest improved biocompatibility as compared to CPDF, others do not unequivocally support this notion (62,63). Amino acids induce mesothelial NO production, a factor involved in neoangiogenesis (64), increase effluent IL-6 concentrations, a surrogate marker

of inflammation, and suppress leukocyte recruitment in rats (65). Long-term dialysis in rats, however, revealed only minor peritoneal changes and preserved ultrafiltration capacity, similar to double chamber PD fluid (62).

Proliferation of PMC is a precondition for an intact mesothelial cell layer (11). Migration, and thus wound-healing capacity, has been shown to depend on the PDF type administered (66). Chronic PD with conventional PDF results in mesothelial cell layer denudation (2) and deterioration of membrane transport characteristics (67). Switching to low GDP fluids, solute transport increases effluent CA125 concentrations 2- to 3-fold (10,26), indicating recovery of the mesothelial cell layer, albeit this has not been proven histologically. Whether the improved preservation of peritoneal membrane function with low GDP fluid (68,69) and the improved outcome as suggested by registry data (70) are also attributable to the preservation of the mesothelium is unknown at present.

Our *in vitro* analysis has important limitations which deserve commenting. *In vivo*, PMC are not only exposed to PDF but also influenced by various other cells present on the basolateral side of the PMC. Coincubation experiments have revealed different findings, e.g. regarding neoangiogenesis capacity of endothelial cells when coincubated with PMC (71). Downregulation of cell cycle mechanisms and thus proliferation capacity may in part be related to the experimental conditions; comparison to whole genome profiles of the peritoneal membrane obtained from patients on PD is required. Still, the huge, up to 10-fold differences in the number of genes regulated by the different PDF indicate a substantial impact of PDF composition on PMC proliferation capacity. While BPDF both up- and downregulated genes to a similar extent, other PDF mostly lead to a downregulation of genes, with striking *p*-values in GO term annotation analyses of $p < 10^{-35}$ – 10^{-33} for the top three affected cellular processes, cell cycle, cell division, and mitosis. Of note, in the group of upregulated genes, associations with processes such as prostaglandin metabolism and leukocyte recruitment were enriched, albeit at a much lower level of significance ($p < 10^{-5}$ – 10^{-4}), pointing toward additional mechanisms relevant in the course of the peritoneum's adaptation to PD.

In conclusion, our large-scale whole genome approach demonstrates a marked impact of the PDF composition on PMC gene regulation. Whereas high GPD and icodextrin-based PDF substantially interfere with the expression of cell cycle and cell division genes and mitigate PMC proliferation, low GDP fluids exert only minor gene regulation and preserve proliferation capacity of PMC, i.e. of cells essential for peritoneal membrane homeostasis.

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