

# Prolonged Exposure to Glucose Degradation Products Impairs Viability and Function of Human Peritoneal Mesothelial Cells

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**Abstract.** Bioincompatibility of peritoneal dialysis fluids (PDF) has been linked to the presence of glucose degradation products (GDP). Previous experiments have shown that short-term exposure to several GDP at concentrations found in commercially available PDF had no significant effect on human peritoneal mesothelial cells (HPMC). During continuous ambulatory peritoneal dialysis, however, cells are continually exposed to GDP for extended periods of time. Thus, the impact of GDP on HPMC during long-term exposure was assessed. HPMC were cultured for up to 36 d in the presence of 6 identified GDP (acetaldehyde, formaldehyde, furaldehyde, glyoxal, methylglyoxal, and 5-HMF) at doses that reflect their concentrations in conventional PDF. At regular time intervals, the ability of HPMC to secrete cytokines (interleukin-6 [IL-6]) and extracellular matrix molecules (fibronectin) was evaluated. In addition,

cell viability, morphology, and proliferative potential were assessed. Exposure to GDP resulted in a significant reduction in mesothelial IL-6 and fibronectin release. Approximately 80% of this decrease occurred during the first 12 d of the exposure and was paralleled by a gradual loss of cell viability and development of morphologic alterations. After 36 d of exposure, the number of cells in GDP-treated cultures was reduced by nearly 60%. However, GDP-treated cells were able to resume normal proliferation when transferred to a normal GDP-free medium. HPMC viability and function may be impaired during long-term exposure to clinically relevant concentrations of GDP, which suggests a potential role of GDP in the pathogenesis of peritoneal membrane dysfunction during chronic peritoneal dialysis.

One of the most unfavorable features of currently used peritoneal dialysis fluids (PDF) is the presence of glucose degradation products (GDP) (1). These compounds result from breakdown of glucose during autoclaving of PDF (2). However, a few of these GDP have been identified. They are mainly low-molecular-weight aldehydes, as exemplified by 5-hydroxymethylfuraldehyde (5-HMF) (3). The key role of high temperatures in precipitating GDP formation is convincingly demonstrated by the fact that GDP are practically absent from PDF sterilized by filtration (3–5).

Several studies have shown the evidence of heat-sterilized PDF being considerably more cytotoxic under *in vitro* conditions compared with filter-sterilized PDF (reviewed in Ref. [6]). Indeed, it has been demonstrated that furaldehyde, acetaldehyde, formaldehyde, 5-HMF, glyoxal, and methylglyoxal

had a potential to inhibit growth either of murine L929 fibroblasts (7) or (to an even greater extent) of human peritoneal mesothelial cells (5). However, these studies also showed unequivocally that if the six investigated aldehydes were tested separately at clinically relevant doses, *i.e.*, corresponding to concentrations actually detected in PDF, their impact was minimal. Furthermore, the addition of these six aldehydes to filter-sterilized PDF did not increase their toxicity to the level of autoclaved solutions. These results may suggest that the contribution of identified GDP to bioincompatibility of heat-sterilized PDF is rather limited. On the other hand, most experiments performed to date have assessed the impact of GDP during short-term exposure periods (up to 72 h) (6). It is therefore conceivable that cells will sustain injury, even from low and apparently nontoxic concentrations of aldehydes, if exposed to GDP for longer periods of time. Such a scenario would be of particular relevance to the situation of clinical continuous ambulatory peritoneal dialysis (CAPD), in which the peritoneum is continually exposed to GDP-containing solutions for months and years. The structure most likely to receive a potential impact from GDP is the peritoneal mesothelium, which forms an interface between the dialysate and the internal milieu. Thus, in this study, we set out to establish an experimental model to investigate the effects of chronic exposure to GDP on human peritoneal mesothelial cells *in vitro*.

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## Materials and Methods

### Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany). All tissue culture plastics were obtained from Sarstedt Inc. (Newton, NC).

### Human Peritoneal Mesothelial Cells

Human peritoneal mesothelial cells (HPMC) were isolated from the specimens of omentum obtained from consenting nonuremic patients undergoing elective abdominal surgery. Cells were isolated and characterized as described elsewhere (8,9). HPMC were propagated in the M199 culture medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), hydrocortisone (0.4  $\mu$ g/ml), and 10% vol/vol fetal calf serum (FCS) (Life Technologies BRL, Eggenstein, Germany). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

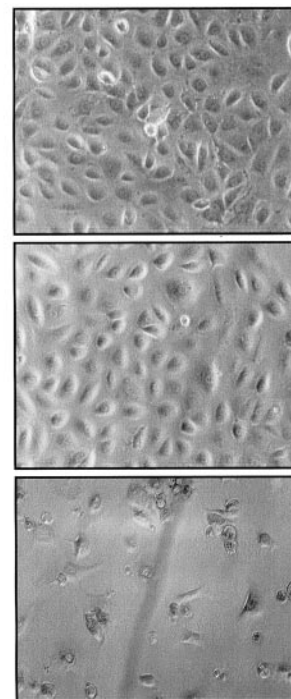
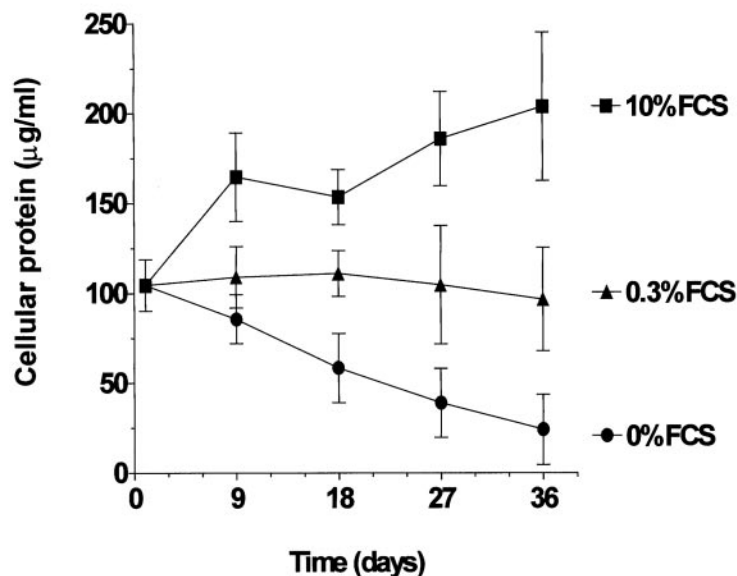
All experiments were performed with cells only from the first three passages because later subcultures contained an increasing number of senescent cells (8). HPMC were plated into multiwell clusters and grown until confluence. The standard medium containing 10% FCS was then replaced with the medium supplemented with 0.3% FCS. Reduction in FCS concentration was meant to reduce the nonspecific effects related to the presence of serum and emphasize, hypothetically, the impact exerted by the GDP. In preliminary studies, we determined the lowest concentration of FCS at which HPMC could be safely cultured throughout the whole experimental period. These experiments demonstrated that HPMC monolayers could be maintained for at least 36 d at FCS concentrations as low as 0.3% without significant alterations in cell morphology and with no apparent reduc-

tion in cell protein content, which indicates a balanced protein turnover (Figure 1). Lower FCS concentrations were not sufficient to maintain HPMC in a viable state. On the other hand, exposure of apparently nonproliferating HPMC monolayers to higher doses of FCS promoted deposition of matrix proteins in culture wells, a situation that did not allow us to make a direct correlation between cell numbers and cell protein levels. Therefore, the 0.3% FCS dose was chosen for further studies.

### Long-Term Exposure to Glucose Degradation Products

GDP examined included acetaldehyde, formaldehyde, 2-furaldehyde, glyoxal, methylglyoxal, and 5-HMF. Formaldehyde and methylglyoxal were provided as 38% and 40% aqueous stock solutions, respectively. The purity of all other products was >98.5%, and that for acetaldehyde, formaldehyde, and 2-furaldehyde met the latest specifications of the American Chemical Society. Glyoxal was used as a stable dihydrate trimer. Working concentrations of GDP were prepared from highly concentrated stock solutions directly before experiments. During the incubation, the culture plates were sealed with Parafilm M (American Can Co., Greenwich, CT), to minimize the potential evaporation of volatile aldehydes (7). HPMC were maintained at 0.3% FCS and exposed to the mixture of GDP added to the culture medium at doses corresponding to the highest concentrations detected in dialysis fluids (3): acetaldehyde, 420  $\mu$ M; formaldehyde, 15  $\mu$ M; 2-furaldehyde, 2  $\mu$ M; glyoxal, 14  $\mu$ M; methylglyoxal, 23  $\mu$ M; and 5-HMF, 30  $\mu$ M.

Control and GDP-treated cells were fed every 3 d and maintained in culture for a total period of 36 d. At defined time intervals (every 12 d), HPMC were exposed to fresh experimental media in the



**Figure 1.** Effect of fetal calf serum (FCS) on human peritoneal mesothelial cells (HPMC) morphology and total cellular protein. HPMC monolayers were maintained at different concentrations of FCS. At the time points indicated, cells were washed, solubilized, and assessed for total protein, as described in the Materials and Methods section. Data are presented as mean values ( $\pm$ SD) obtained from six experiments, with cells isolated from separate donors. Representative microphotographs of HPMC cultures were taken after 36 d of incubation with 10% FCS (top), with 0.3% FCS (middle), or without FCS (bottom). Magnification,  $\times 40$ .

presence or absence of interleukin-1 $\beta$  (IL-1 $\beta$ ) (100 pg/ml; R&D Systems, Wiesbaden, Germany). After 24 h, the supernates were collected, centrifuged at 12,000  $\times$  *g* to remove any cellular debris, and stored at  $-70^{\circ}\text{C}$  until assayed for IL-6 and fibronectin. Cell monolayers were washed with PBS and solubilized with 0.1% (vol/vol) Triton X-100; the lysates were measured for total protein and fibronectin. Cell viability was assessed as described below.

### MTT Assay

Cell viability was assessed by measuring their ability to metabolize the MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolinum bromide) (10). The assay was performed essentially as described elsewhere (5).

### Immunoassays

IL-6 concentrations in HPMC-derived supernates were measured with a specific enzyme-linked immunosorbent assay (ELISA). The assay was designed with the use of an ELISA-matched antibody pair (R&D Systems) and performed according to manufacturer's instructions. Sensitivity of the system was 2 pg/ml.

### Fibronectin Measurements

For fibronectin determination by ELISA, multiwell plates (Nunc, Nalge Nunc International, Rochester, NY) were coated with 2.5  $\mu\text{g}/\text{ml}$  of rabbit anti-human fibronectin antibody (Dako Diagnostika, Hamburg, Germany) in 50 mM  $\text{Na}_2\text{CO}_3$  and then blocked with 10% Roti-Block (Carl Roth, Karlsruhe, Germany). After that, the samples and standards of human plasma fibronectin (Sigma) were added and incubated at  $4^{\circ}\text{C}$  overnight. Captured molecules were detected with sheep anti-human fibronectin antibody coupled to horseradish peroxidase (0.5  $\mu\text{g}/\text{ml}$ ; Biogenesis/Quartett, Berlin, Germany). The color was developed after the exposure to the substrate solution of tetramethylbenzidine and  $\text{H}_2\text{O}_2$  (ICN Biomedicals, Rzeszow, Poland). The reaction was stopped with 2 N  $\text{H}_2\text{SO}_4$ , and the optical density was measured at 450 nm with a reference wavelength of 540 nm. Sensitivity of the assay was 0.31 ng/ml.

### Cell Protein Measurements

Total cellular protein in solubilized HPMC monolayers was analyzed with the BCA protein assay (Pierce, Rockford, IL), with the use of bovine serum albumin as the standard. Repeated cell counts revealed that 1  $\mu\text{g}$  of HPMC protein corresponded to (mean  $\pm$  SD)  $2.08 \pm 0.89 \times 10^3$  cells under control conditions and to  $2.14 \pm 1.32 \times 10^3$  cells exposed to GDP for 36 d ( $n = 11$ ). Because the difference was not significant, the results derived from control and GDP-treated cultures were calculated per 1  $\mu\text{g}$  of cell protein.

### Proliferation Studies

The number of cells in HPMC monolayers was assessed after 36 d of incubation in either control or GDP-containing medium. Cells were harvested with solution of 0.05% (wt/vol) trypsin and 0.02% (wt/vol) ethylenediaminetetraacetic acid (EDTA) and counted directly by use of a Neubauer chamber. After that, HPMC were suspended in standard GDP-free medium, seeded at equal density of  $2.5 \times 10^4/\text{cm}^2$  into new culture plates, and stimulated with 10% FCS to assess their proliferative potential. After 8 h, the nonadherent cells were removed by gentle low-shear wash with culture medium, and the remaining adherent cells were detached with trypsin-EDTA solution and counted. In parallel wells, HPMC were allowed to proliferate for the

next 24 h, after which the number of cells was measured again. Mean population doubling time (mPDT) was calculated from the equation

$$\text{mPDT} = \Delta T \times \log_2 / \log (C_t / C_o),$$

where  $\Delta T$  represents the incubation time,  $C_o$  is the number of cells at the beginning of incubation, and  $C_t$  is the number of cells at the end of incubation (11).

### Statistical Analyses

All statistical analyses were performed by use of GraphPad Prism 3.00 software (GraphPad Software Inc., San Diego, CA). The comparisons were made with nonparametric tests for paired data (the Wilcoxon test or repeated-measures ANOVA followed by the Friedman statistic, where appropriate).  $P < 0.05$  was considered to be significant. Box and whisker graphs represent the median, 25th and 75th percentiles, and range of the data.

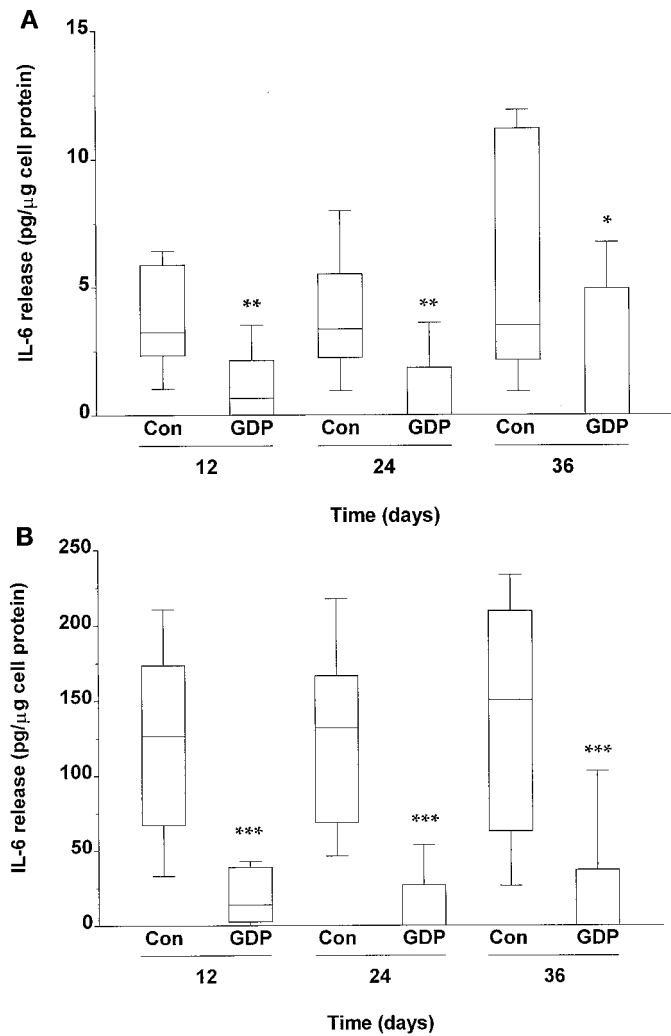
## Results

Incubation of HPMC in the presence of GDP led to a time-dependent reduction in the ability of HPMC to release IL-6, either constitutively or in response to IL-1 $\beta$  stimulation (Figure 2). After 12 d of exposure, the basal and IL-1 $\beta$ -driven IL-6 secretion was reduced by a mean of 42.5% and 83.9%, respectively ( $n = 11$ ). Similar degrees of inhibition were observed after 24 and 36 d.

In control HPMC cultures, the average production of fibronectin during a 24 h period was (mean  $\pm$  SD)  $2.14 \pm 1.61$  ng/ $\mu\text{g}$  cell protein (25 measurements in  $n = 9$  cell lines). Of this amount,  $92.5 \pm 4.2\%$  was found in the supernates, whereas only  $7.5 \pm 4.2\%$  was detected in cell lysates. Exposure to GDP did not have a major impact on the fraction of intracellular fibronectin (Figure 3A). In contrast, incubation with GDP resulted in a significant decrease in the release of fibronectin. The mean inhibition recorded after 12 d was 82.8% compared with the control and remained at this level over the whole time course studied (Figure 3B). Exposure to IL-1 $\beta$  (100 pg/ml) changed neither the total amount of fibronectin produced nor its distribution in HPMC cultures. The effect of GDP under these conditions resembled that observed in the absence of IL-1 $\beta$  (data not shown).

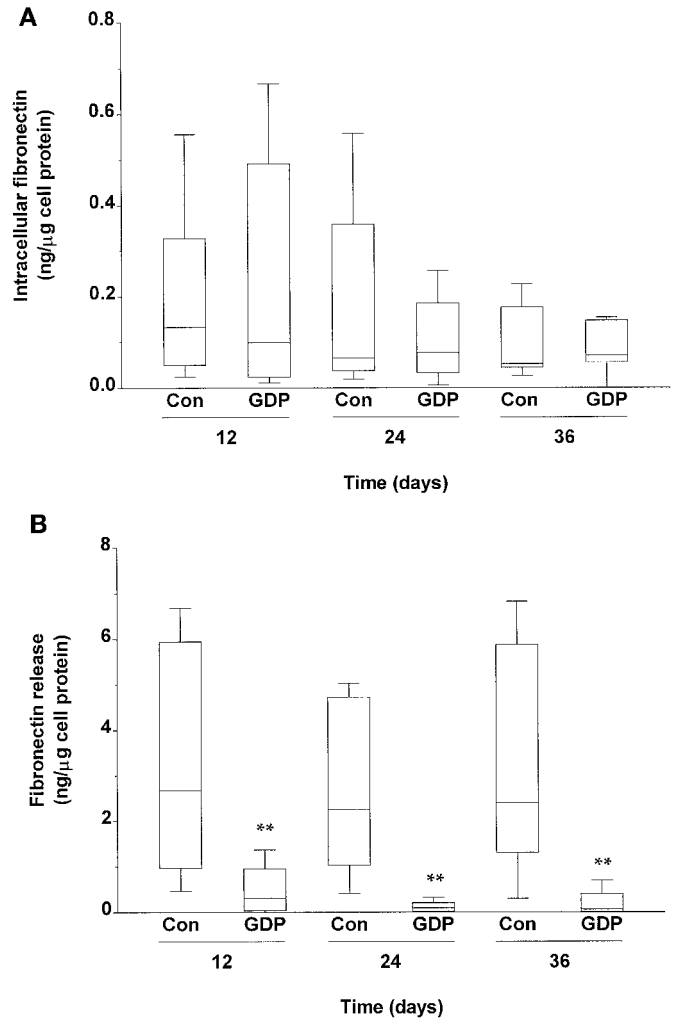
The presence of GDP in the incubation media decreased the number of cells in HPMC monolayers assessed after 36 d of exposure. The number of cells in cultures exposed to GDP was (mean  $\pm$  SD,  $\times 10^3$ )  $238.3 \pm 147.6$ , compared with  $547.3 \pm 234.0$  in the controls ( $n = 10$ ,  $P < 0.01$ ). When the GDP-treated cells were suspended in fresh GDP-free culture medium and seeded into new culture flasks, the number of cells that had adhered to plastic during an 8-h observation period was still lower than that of control cells (Figure 4); plating efficiency was reduced from (mean  $\pm$  SD)  $85.8 \pm 28.3\%$  in the control group, compared with  $61.4 \pm 25.2\%$  in the GDP group ( $n = 8$ ,  $P < 0.05$ ). However, the subsequent proliferation of GDP-treated cells in response to stimulation with 10% FCS was not different from that observed in control HPMC. The calculated mean population-doubling time was (mean  $\pm$  SD)  $58.9 \pm 12.7$  h for control cells and  $60.9 \pm 56.5$  h for HPMC that had previously been exposed to GDP.

The timescale of the decrease in IL-6 and fibronectin release



**Figure 2.** Effect of prolonged exposure to glucose degradation products (GDP) on the release of interleukin-6 (IL-6) by HPMC. Cells were incubated in either control medium or in the presence of a defined mixture of GDP. The release of IL-6 during a 24-h period was assessed at the time intervals indicated. Data represent constitutive (A) and IL-1 $\beta$ -induced (B) secretion of IL-6 recorded in 11 HPMC cultures derived from separate donors. Box and whisker plots represent the median, 25th and 75th percentiles, and range of the data. Asterisks represent statistically significant differences, with \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

suggested that the most significant impairment of HPMC function occurred within the first 2 wk of the exposure to GDP. Thus, we have assessed viability and morphology of HPMC at more frequent intervals within this time frame. Exposure to GDP resulted in a progressive loss of cell viability, as measured by the ability to metabolize the MTT salt (Figure 5). A statistically significant decrease was evident by day 10, and after 14 d the viability of GDP-treated HPMC was only (mean  $\pm$  SD) 33.0  $\pm$  13.8% of that recorded in control cells ( $n = 6$ ). Reduction in viability was associated with considerable alterations in cell morphology, although the intensity of these changes varied between cell lines. Figure 6 documents the



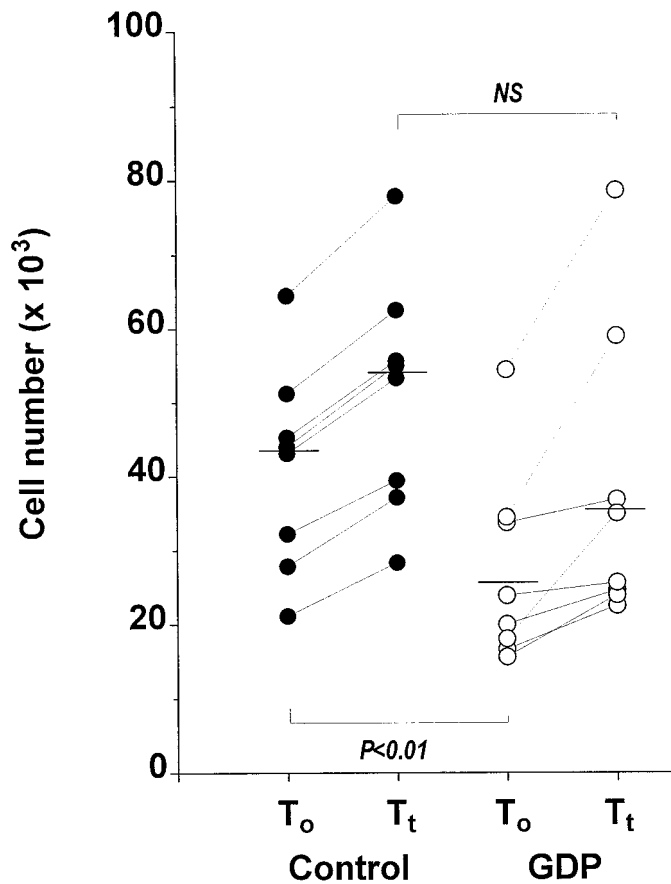
**Figure 3.** Effect of GDP on the production of fibronectin by HPMC. Cells were incubated in either control medium or in the presence of a defined mixture of GDP. The release of fibronectin during a 24-h period was assessed at the time intervals indicated. Data represent either intracellular (A) or secreted (B) fibronectin detected in ten experiments with HPMC from different donors. Box and whisker plots represent the median, 25th and 75th percentiles, and range of the data. Asterisks represent statistically significant differences, with \*\* $P < 0.01$ .

serious injury to HPMC that resulted from the exposure to GDP.

### Discussion

In an attempt to evaluate the biologic impact of GDP present in dialysis solutions, several previous studies have compared the effect of heat-sterilized and filter-sterilized PDF on cell proliferation (7,12–15). By definition, these studies could span only a limited time frame—that is, the phase of exponential cell growth. In other experimental protocols, the release of cytokines or reactive oxygen species in response to GDP-containing PDF was measured again after short-term pre-exposure periods (1,15–17). Furthermore, only two studies—one by Wieslander *et al.* (7) of the murine L929 fibroblast cell line

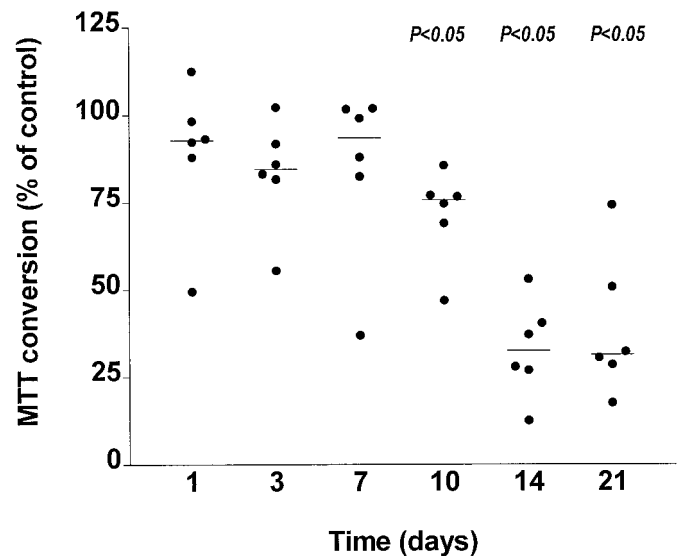




**Figure 4.** Serum-stimulated proliferation of GDP-treated cells. HPMC cultures were incubated for 36 d in the presence or absence of GDP. After that, the equal numbers of cells were transferred to the GDP-free environment and stimulated with 10% FCS, as described in the Materials and Methods section. The number of proliferating cells was assessed again after 8 ( $T_0$ ) and 24 ( $T_t$ ) h. Data were obtained from eight separate HPMC cultures. Horizontal bars represent the median values.

and one by our group (5), with human peritoneal mesothelial cells—have assessed the direct effect of GDP identified in PDF. These studies also concentrated on acute consequences of the exposure to GDP. In this investigation, we assessed, for the first time, viability and function of HPMC exposed chronically to GDP.

To this end, it was shown that HPMC monolayers could be maintained *in vitro* for up to 36 d in the presence of as little as 0.3% FCS. This reduced FCS concentration was sufficient to support baseline viability but at the same time offered only minimal protection against GDP-mediated toxicity. A previous study (5) revealed the importance of choosing for GDP testing primary cultures of HPMC rather than established animal cells lines, because the same doses of aldehydes impaired HPMC viability and function to a significantly greater degree compared with L929 cells. Furthermore, the use of primary cultures of HPMC enabled the demonstration of the real extent of biologic variability. The mesothelial production of inflammatory mediators and extracellular matrix molecules, exemplified



**Figure 5.** Time effect of GDP on viability of HPMC. Confluent HPMC cultures were exposed to either control or GDP-containing medium. At the time points indicated, cell viability was assessed with the MTT test, as described in the Materials and Methods section. Results were obtained from six separate experiments, with cells isolated from different donors and expressed as percentages of respective control values. Horizontal bars represent the medians.

by IL-6 and fibronectin, respectively, were chosen as relevant parameters of cell function.

The key finding of this study was the fact that GDP, at doses that proved to be nontoxic in short-term experiments (5), significantly impaired viability and function of HPMC during prolonged exposure. Long-term incubation of HPMC with a defined mixture of GDP led to significant reduction in the release of both IL-6 and fibronectin proteins. The greatest decrease in HPMC activity occurred during the first 2 wk. The effect could partially be attributed to the gradual decline in HPMC viability, which was clearly visible within this time period.

Reduced cell viability could also explain the progressive loss of cells from HPMC monolayers. In addition, even those GDP-treated cells that remained in a viable state and were later suspended in normal medium displayed reduced plating efficiency. Normally, HPMC attach rapidly (within 15 to 90 min) to either plastic surfaces (Witowski and Breborowicz, personal observations; (18) or extracellular matrix proteins, including collagen I and IV, laminin, fibronectin, and vitronectin (19). Prolonged treatment with GDP appeared to impair the adhesive capacity of HPMC such that, after as long as 8 h after the seeding, a substantial percentage of cells remained unattached. One could speculate that the reduced ability of HPMC to release and deposit fibronectin might contribute both to this effect and to detachment of cells from the monolayers. Fibronectin can be detected in large quantities in body fluids, including CAPD effluent (20–23). The greatest portion of fibronectin in the peritoneal cavity is probably derived from the circulation (23). However, it is also produced by both mesothe-

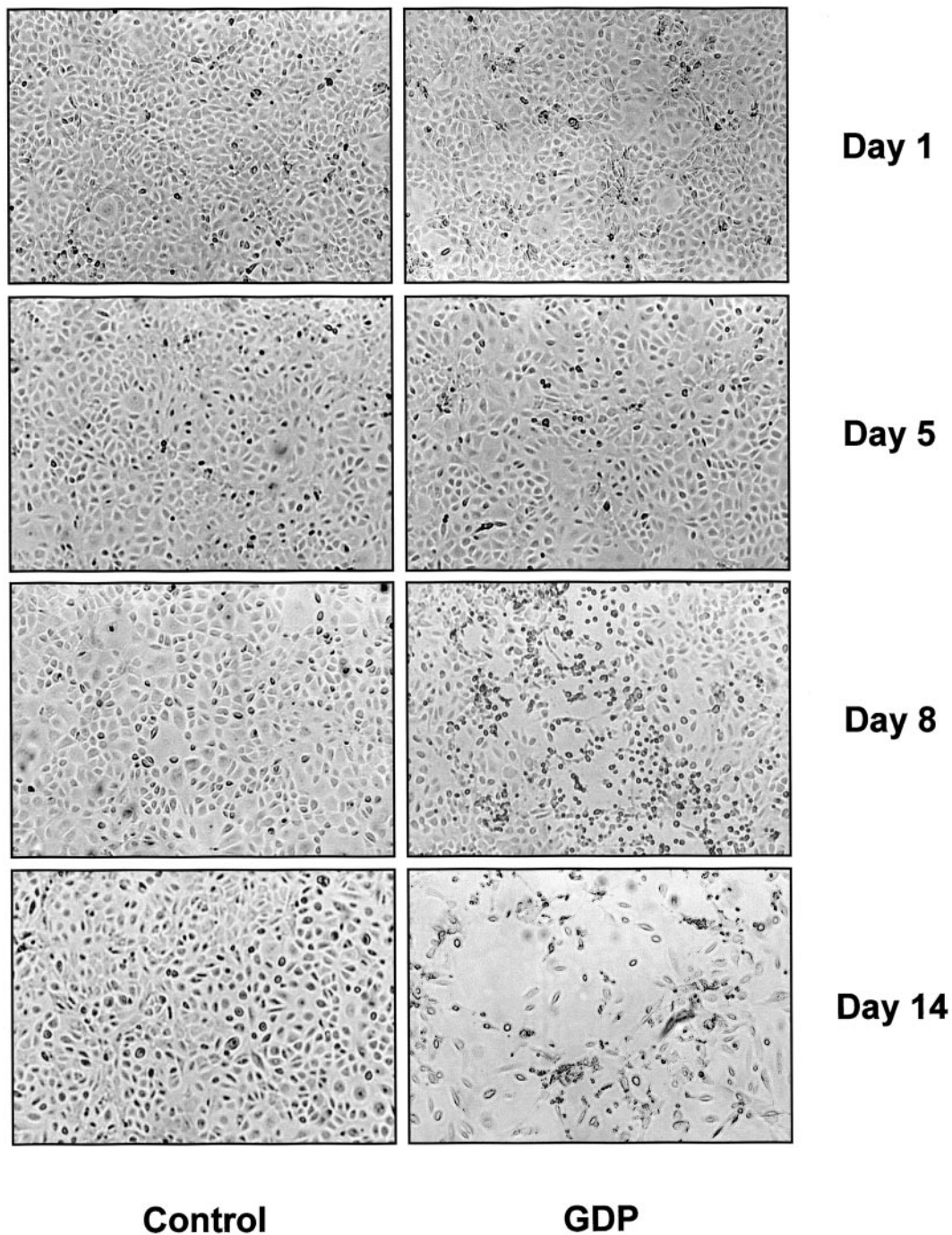


Figure 6. Time course of morphological changes in HPMC treated with GDP. Representative microphotographs of HPMC treated with control or GDP-containing medium. Magnification,  $\times 40$ .

lial cells (8,24,25) and peritoneal macrophages (22). Leavesley *et al.* (19) demonstrated that fibronectin greatly supported firm attachment of HPMC under *in vitro* conditions. Increased adherence of HPMC to fibronectin-coated surfaces has also been documented in several other studies (26,27).

Mesothelial cells can be found in spent dialysate drained from CAPD patients (18,19) but are usually absent from the peritoneal fluid obtained from healthy subjects undergoing diagnostic laparoscopy (28). These observations suggest that

the adhesion of HPMC may be compromised by dialysis fluid components and/or shear stress related to repeated infusions of the PDF. It remains to be determined to what extent GDP-mediated decrease in mesothelial fibronectin production contributes to continuous shedding of HPMC into the dialysate (29). Early clinical studies with newly formulated PDF with reduced GDP content did not provide direct data on the number of HPMC recovered from the effluent. However, they documented increased intraperitoneal levels of CA<sub>125</sub>, which is

believed to reflect mesothelial cell mass and—indirectly—better preservation of HPMC in the peritoneum (30–33).

One of the limitations of this study was the use of only six GDP out of many more present in PDF but not yet identified. One of those compounds was recently defined as 3-deoxyglucosone (34). However, we decided not to include this in our GDP mixture because we wanted to relate the results to those obtained in earlier experiments with short-term exposure periods (5). In addition, our preliminary observations indicate that 3-deoxyglucosone at doses as high as 500  $\mu\text{M}$  exerts no major effect on HPMC, at least in terms IL-6 synthesis, during a 6-d exposure period (35).

A comparison of acute and chronic studies convincingly shows that time of the exposure is a crucial factor that determines the cytotoxic effect of GDP on HPMC. Assessment of GDP-mediated toxicity based solely on short-term effects may therefore significantly underestimate the deleterious potential of GDP toward the peritoneum. One cannot also exclude interactions between GDP and dialysate glucose. It is well recognized that glucose may retard HPMC growth and viability *in vitro* (36,37). Our observations indicate, however, that over the period of 6 wk, GDP-mediated inhibition of HPMC function and viability was not significantly modulated by glucose at doses up to 30 mM (Witowski and Breborowicz, unpublished data). Recent evidence has shown that, in addition to PDF-derived carbonyl compounds, patients undergoing CAPD may receive an additional insult from increased generation of endogenous glucose derivatives triggered by abnormal biochemistry in uremia (38). On the other hand, some of the GDP appear to be rapidly cleared from the peritoneal cavity (39), by either absorption into the circulation or by the interaction with peritoneal cells. Thus, the extent of exposure of peritoneal tissues to GDP may vary among patients. Better understanding of how GDP affect the mesothelial cell biology, together with technical advances aiming at reducing the level of GDP in dialysis solutions, may help to maintain the dialyzing function of the peritoneum and prolong the survival of the CAPD technique.

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