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Glucose degradation products (GDP) retard remesothelialization independently of D-glucose concentration

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Glucose degradation products (GDP) retard remesothelialization independently of D-glucose concentration.

Background. Glucose degradation products (GDP) present in heat-sterilized dialysis fluids are thought to contribute to cellular dysfunction and membrane damage during peritoneal dialysis. To examine the effects of specific GDP on the remesothelialization process, the impact of conventional and low GDP peritoneal dialysis solutions, D-glucose, and individual GDP in a scratch-wounding model was assessed.

Methods. Scratch (0.5 to 0.6 mm)-wounded human peritoneal mesothelial cells (HPMC) were treated, at pH 7.4, with either (1) control medium (M199), (2) laboratory-prepared heat or filter-sterilized solutions, (3) 10% to 80% vol/vol solution of Gambrosol® or Gambrosol-trio® (1.5% and 4.0% glucose), (4) D-glucose (5 to 80 mmol/L), or (5) individual or combined GDP [acetaldehyde, formaldehyde, glyoxal, methylglyoxal, 3-deoxyglucosone (3-DG), 5-hydroxy methylfufural (5-HMF), or 3,4-di-deoxyglucosone-3-ene (3,4-DGE)]. Wound closure was recorded by time-lapse photomicroscopy.

Results. In untreated HPMC, the rate of wound closure was linear and the process was complete by 18.4 ± 3.6 hours (N = 16). In wounded HPMC exposed to dilutions of heat-sterilized but not filtered laboratory solutions (1.5% or 4.0% glucose, pH 7.4), remesothelialization was significantly retarded (P = 0.04 and P = 0.009 vs. M199, respectively). In Gambrosol®, remesothelialization was significantly retarded in both 1.5% and 4.0% solutions. In contrast in Gambrosol-trio®–treated HPMC, this rate was not significantly reduced in either 1.5% or 4.0% glucose peritoneal dialysis fluids. Remesothelialization was dose-dependently retarded in HPMC exposed to 3,4-DGE (>10 μmol/L), formaldehyde (>5 μmol/L) but not by exposure to the other GDP tested even at 5 times the concentration present in low glucose solutions. The rate of remesothelialization was not significantly altered by exposure to D-glucose concentrations up to 80 mmol/L.

Conclusion. These data identify that the formaldehyde and 3,4-DGE present in heat-sterilized peritoneal dialysis solutions are important in reducing mesothelial cell regeneration. Specifically targeting their removal may have major benefits in preserving the mesothelium during long-term peritoneal dialysis.

Key words: mesothelium, glucose degradation products, peritoneum, formaldehyde, 3,4-di-deoxyglucosone-3-ene.

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The mesothelial cells lining the peritoneal cavity play a central role in maintaining peritoneal membrane integrity and contributing to peritoneal host defense and homeostasis [1]. During peritoneal dialysis, the mesothelium is continuously exposed to unphysiologic peritoneal dialysis fluids. Continuous mesothelial injury occurs as evidenced by increased numbers of exfoliated cells present in dialysis effluent, by decreased markers of mesothelial cell mass in long-term peritoneal dialysis patients and by recent evidence from the Biopsy Registry identifying denudation in a large percentage of parietal peritoneal samples [2, 3]. Mesothelial cell injury is exacerbated during episodes of peritonitis [4, 5]. These data suggest that continuous mesothelial cell turnover is a feature of the dialyzed peritoneal cavity and that regeneration of these cells is an important feature in maintaining membrane integrity. Under normal conditions, the mesothelium has the ability to repair damaged areas by a processes involving both cell proliferation, migration, and reseeding of the denuded areas [6–9]. In vitro data suggest that remesothelialization is regulated by a number of growth factors, including epidermal growth factor (EGF), that may be endogenously produced following cell injury [10, 11]. These data are supported by evidence of the ability of mesothelial cells to repair wounded areas in the absence of exogenous growth factor addition [8].

There is now considerable evidence that components of conventional heat-sterilized peritoneal dialysis fluids can significantly modulate mesothelial cell function. While low pH and high lactate concentrations have been shown to reduce parameters of cell activation and viability,

more recent data suggest that glucose concentrations, hyperosmolality, and lactate may be important mediators of mesothelial cell activation contributing to profibrogenic and proinflammatory phenotype [12–17]. To maintain sterility, conventional peritoneal dialysis solutions are autoclaved and, although this process is performed at acidic pH to prevent the caramelization of glucose, it results in the formation of breakdown products of glucose whose concentrations also increase with storage. Many of the glucose degradation products (GDP) present in peritoneal dialysis fluids have now been identified and their biologic reactivity characterized in various assay systems, including both acute- and chronic-exposure models using mesothelial cells [18–21]. These data suggest that individual GDP have different biologic activities resulting in both inhibition and in some cases activation of specific mesothelial cell functions, including proliferation, cytokine and growth factor synthesis, and cell viability [20, 22, 23]. In addition to their direct ability to modulate cell function, their potential spectrum of biologic activity is further enhanced by their ability to contribute directly and rapidly to the process of advanced glycation end product (AGE) deposition [24, 25], formation of which have been implicated in mesothelial cell activation [26] and contributing to the morphologic alterations in the peritoneal membrane [27, 28].

The identification of the potential deleterious impact of GDP exposure has resulted in the development of peritoneal dialysis fluids specifically engineered to reduce their concentrations [29–31]. These multichambered bag systems allow glucose to be sterilized separately from the other electrolytes at low pH (pH 3 to 4)) to reduce GDP formation to a minimum [32]. Clinical studies using these "low GDP" solutions have identified reduced impact on cell function and evidence of increased mesothelial mass following their continuous use [29, 31, 33, 34].

In the present study, we have examined the specific impact of GDP on the endogenous remesothelialization process following wounding. Our data suggest that at clinically relevant concentrations (i.e., those present in low glucose conventional heat-sterilized peritoneal dialysis fluids) that formaldehyde and 3,4-di-deoxyglucosone-3-ene (3,4-DGE) impair the mesothelial cell repair, independent of effects on cell viability or D-glucose concentration. These observations suggest that targeted reduction of specific GDP species may be important in preserving mesothelial cell and peritoneal membrane integrity during long-term peritoneal dialysis.

METHODS

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, Ltd. (Poole, Dorset, UK). All tissue culture plastics were Falcon®, obtained from Becton Dickinson Labware (Cowley, Oxford, UK).

Isolation and culture of human peritoneal mesothelial cells

Human peritoneal mesothelial cells (HPMCs) were isolated by enzymatic digestion of human omentum of consenting patients undergoing abdominal surgery as previously described [35, 36]. Cells were maintained in Medium 199 (M199) containing 5 mmol/L D-glucose (Gibco BRL, Life Technologies, Ltd., Paisley, Scotland) containing 10% fetal calf serum (FCS) (Hyclone, Aalst, Belgium) and supplemented with penicillin 100 U/mL, streptomycin 100 μg/mL, glutamine 2 mmol/L (Gibco BRL, Life Technologies, Ltd.), insulin 5 μg/mL, transferrin 5 μg/mL, and hydrocortisone 0.4 μg/mL. Confluent cells were split at a ratio of 1:3 and second-passage cells were used in all experiments. All solutions were free of endotoxin as per manufacturers' information.

Scratch wounding of HPMC monolayers

HPMC were grown to confluence in 24-well plates and growth arrested for 48 hours in serum-free M199. HPMC monolayers were wounded by a modification of the method of Yung and Davies [8]. The monolayer was wounded by a single gentle scrape with a sterile, 0.5 mm diameter round sterile stainless steel rod without damaging the base of the well. This resulted in a linear "wound" of between 0.5 and 0.6 mm. The average diameter of the wounded area was 0.55 ± 0.06 mm ($N = 64$ separate wells). In cases where damage to the well was visible, these were not used for data analysis. The monolayers were then washed (with warm M199) to remove detached cells and cellular debris before incubation with test or control solutions for defined time periods up to 72 hours. Scratch wounding resulted in the removal of $<4\%$ of the HPMC monolayer from each well.

Assessment of remesothelialization by time-lapse photomicroscopy

The denuded area in each well was identified microscopically and the coordinates recorded for subsequent data capture. Remesothelialization was continuously monitored using an Axiovert 100M inverted microscope fitted with a computer-controlled XY automated scanning stage and incubator. The humidified incubator was maintained at 37°C and 5% CO_2 with a heated insert and vectorial airflow (Carl Zeiss, Oberkochen, Germany). Images were captured from the same position in each well of the 24-well plates, using the 2.5× objective, at 60-minute intervals on an Orca C5985 digital video camera (Hamamatsu Photonics, Hamamatsu City, Japan). Images were analyzed using Openlab version 3.0.8 on a Macintosh G4 computer (Improvision, Ltd., Coventry, UK). The rate of remesothelialization was calculated by

measuring the reduction of the denuded area (in pixels) at 60-minute intervals. Data are presented as μm^2/hour for data converted from pixels following calibration of the system with a graticule (Carl Zeiss).

Preparation of test solutions

Laboratory prepared fluids were of identical composition to standard solutions containing either 1.5% or 4.0% D-glucose (Gambrosol®) (Gambro, Lund, Sweden). These solutions were either heat-sterilized (120°C/20 minutes) or filter-sterilized (0.2μ filter) (Millipore, Watford UK). D-glucose was obtained from Sigma-Aldrich, Ltd. All solutions were stored at 4°C and fresh aliquots were used for each experiment. All solutions had endotoxin levels below the detection limit as assessed by limulus amebocyte lysate (LAL) assay (Sigma-Aldrich, Ltd.).

Commercially available solutions were either conventional lactate-buffered, heat-sterilized fluids (Gambrosol®) containing 1.5% or 4.0% dextrose or those manufactured in a triple-chambered bag (Gambrosol-trio®) (Gambro, Lund, Sweden) containing 1.5% or 4.0% dextrose. For experimental procedures, solutions were diluted to 10% to 80% (vol/vol) with M199 containing 2 mmol/L N-2-hydroxyethylpiperazine-N′-2ethanesulfonic acid (HEPES) (to ensure that the pH was maintained at 7.4 throughout the experiment). All solutions were at a pH of 7.4. D-glucose was diluted in M199/HEPES (pH 7.4) and was used in HPMC exposure experiments at a concentration range between 5 and 80 mmol/L.

Acetaldehyde, formaldehyde, methylglyoxal, and 5-hydroxy methylfufural (5-HMF) were purchased from Sigma-Aldrich, Ltd. Glyoxal was purchased from Merck-Eurolab (Stockholm, Sweden) and 3-deoxyglucosone (3-DG) from Toronto Research Chemicals (Ottawa, Canada). 3,4-di-deoxyglucosone-3-ene (3,4-DGE) was extracted from autoclaved glucose solutions using solid-phase extraction (ENV-columns) (International Sorbent Technologies, Pontypridd, UK) based on previously described methods [37]. GDP were diluted from the stock solutions in M199/HEPES (pH 7.4) and used in wounding experiments either as a mixture of each of the tested GDP (at the concentrations present in 1.5% heat-sterilized peritoneal dialysis fluids) or singly at concentrations from one to five times those reported in commercially available heat-sterilized 1.36% to 1.5% glucose-containing peritoneal dialysis fluids glyoxal (10 to 50 μmol/L), methylglyoxal (12 to 48 μmol/L), acetaldehyde (0.1 to 0.5 mmol/L), 3,4-DGE (10 to 40 μmol/L), formaldehyde (5 to 50 μmol/L), 3-DG (100 to 500 μmol/L), and 5-HMF (1 to 5 μmol/L) [38–40].

Assessment of HPMC viability

HPMC viability was assessed 48 hours following wounding measurement of intracellular adenosine triphosphate (ATP) content as previously described [41].

Assessment of HPMC proliferation

HPMC cell numbers in wounding experiments were assessed by 3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [42]. Based on standard curves of increasing HPMC numbers (10^3 to 10^5/well), there was a direct correlation between HPMC numbers and MTT optical density ($N = 16$, $r = 0.998$, $P < 0.001$). Incubation of HPMC with different test solutions did not affect MTT optical density (data not shown).

Statistical analysis

All experiments were performed in 24-well plates with random assignment of wells to different treatments. All data are presented as mean [±standard error of the mean (SEM)] from five to nine experiments performed in quadruplicate with HPMC isolated from different omental donors. Based on the nonnormal distribution of the data, statistical analysis between two groups (wound areas at the start and end of the observation period) was performed using Wilcoxon signed rank test for related samples (SPSS version 10, SPSS, Inc., Chicago, IL, USA). Differences were considered significant when $P < 0.05$.

RESULTS

Remesothelialization of HPMC

As in previous experiments in 35 mm dishes [8], when confluent HPMC were wounded (0.5 to 0.75mm scratch) in 24-well plates, complete remesothelialization occurred under serum-free conditions and appeared to be dependent on cell migration (as evidenced by no significant increases in MTT values). In these experiments, remesothelialization was linear for the first 12 to 15 hours of the experiment and wound closure was found to be complete within 18.4 ± 3.6 hours ($N = 16$) (Fig. 1). The linearity of the remesothelialization response was maintained regardless of treatment and was only significantly disrupted in the face of significant loss of cell viability. The rate of remesothelialization under serum-free conditions in M199 was median 33.3 (range, 18.6 to 50.7) μm^2/hour ($N = 16$ different cell lines).

Remesothelialization following exposure to laboratory-prepared fluids

In order to examine the importance of GDP in modulating remesothelialization, laboratory-prepared peritoneal dialysis fluids were prepared and either heat- or filter- sterilized. In order to minimize the impact of cell viability in the remesothelialization experiments, preliminary viability experiments (in confluent HPMC) were performed. These identified that dilution of all test solutions 1:4 (25%vol/vol) in M199 avoided significant loss of monolayer viability over a 24-hour period [as assessed

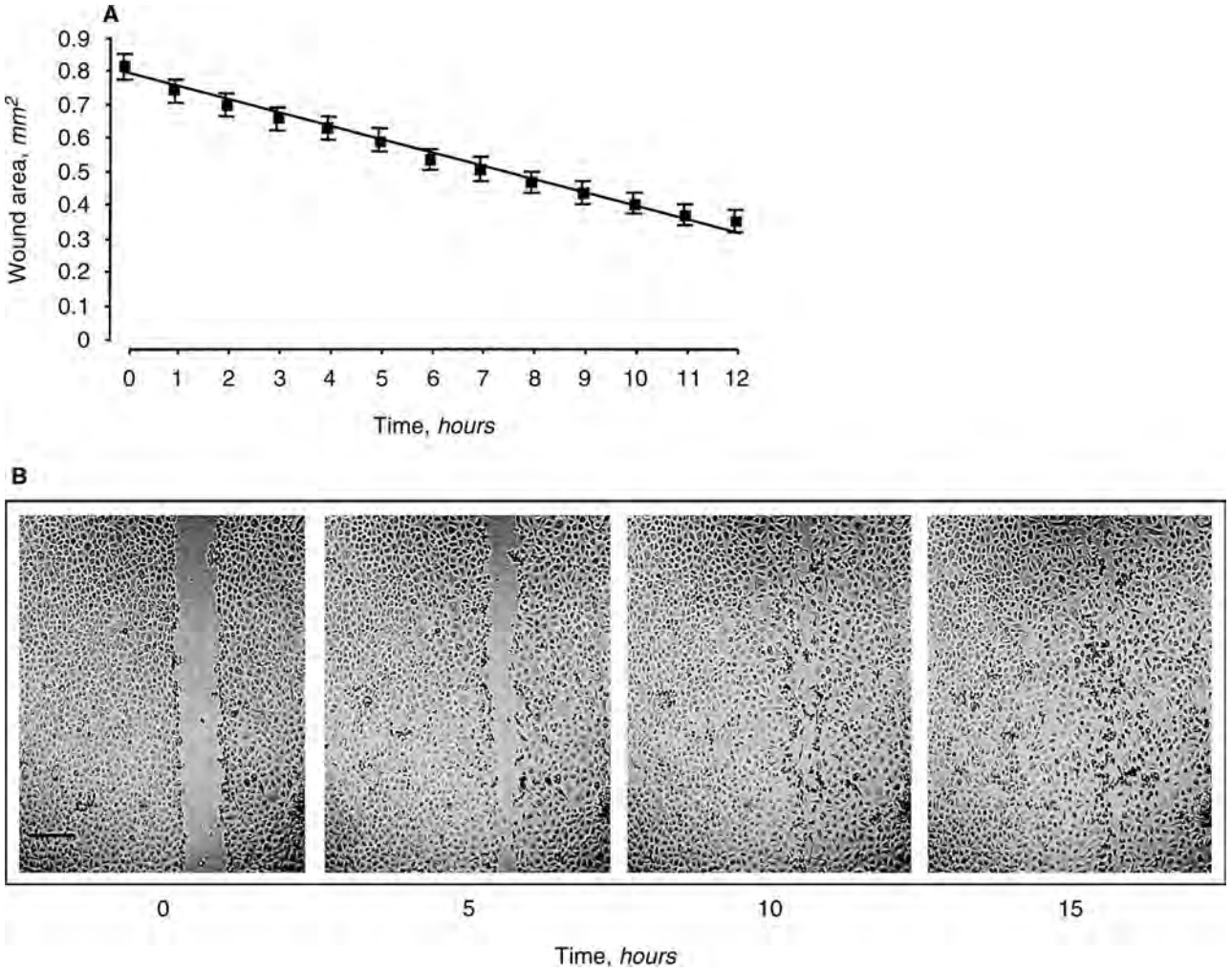

Fig. 1. Time course of remesothelialisation (*A*) and time-lapse photography (*B*) following scratch wounding in human peritoneal mesothelial cells (HPMC) exposed to control medium (M199). Data presented are the mean wound areas (± SEM) at the indicated time points from 16 experiments . Wound healing rate is calculated over the first 12 hours postwounding from the gradient of the line; median rate 33.3 (range, 18.6 to 50.7) μm/hour ($N = 16$ different cell lines). Time-lapse photographs demonstrate linear wound closure accompanied by migration of phenotypically altered cells into the denuded area prior to complete closure by between 10 and 15 hours (representative pictures presented).

by total ATP measurement (data not shown)]. A 25% vol/vol dilution was subsequently used in all HPMC wounding experiments in which laboratory-prepared or commercial dialysis solutions were used.

Exposure of scratch-wounded HPMC to heat-sterilized laboratory-prepared peritoneal dialysis fluids resulted in a significant retardation or failure of remesothelialization (8.8 ± 4.4 μm²/hour and −10.6 ± 4.43 μm²/hour for 1.5% and 4.0% D-glucose peritoneal dialysis fluids, respectively) ($N = 5$; $P = 0.04$ and $P = 0.009$ vs. M199) (Fig. 2A). In contrast, in wounded HPMC exposed to filter-sterilized peritoneal dialysis fluids, remesothelialization proceeded at a normal rate in both 1.5% (24 ± 3.6 μm²/hour) and 4.0% (32 ± 1.5 μm²/hour) and M199 (28 ± 3.0 μm²/hour), respectively) ($N = 6$; P = NS for both vs. M199) (Fig. 2A).

Assessment of cell numbers by MTT assay showed a significant reduction ($N = 6$; $P = 0.04$ for both) in HPMC treated with heat-sterilized fluids compared to filtered fluids and to controls (M199) at the conclusion of the experiment (72 hours) (Fig. 2B).

Remesothelialization following exposure to commercial peritoneal dialysis fluids

Wounded HPMC exposed to 25% vol/vol conventional lactate-buffered peritoneal dialysis fluids (Gambrosol®) of both high and low glucose content showed complete retardation in remesothelialization in both 1.5% and 4.0% Gambrosol® ($N = 6$; $P = 0.04$ and $P = 0.02$ vs. M199, respectively) (Fig. 3A). In Gambrosol-trio®, however, the remesothelialization rate was not different to M199 (34 ± 3.2 μm²/hour, 25.2 ± 9.4 μm²/hour, and 28.1 ± 3.6 μm²/hour for 1.5% Gambrosol-trio®, 4.0% Gambrosol-trio®, and M199, respectively)

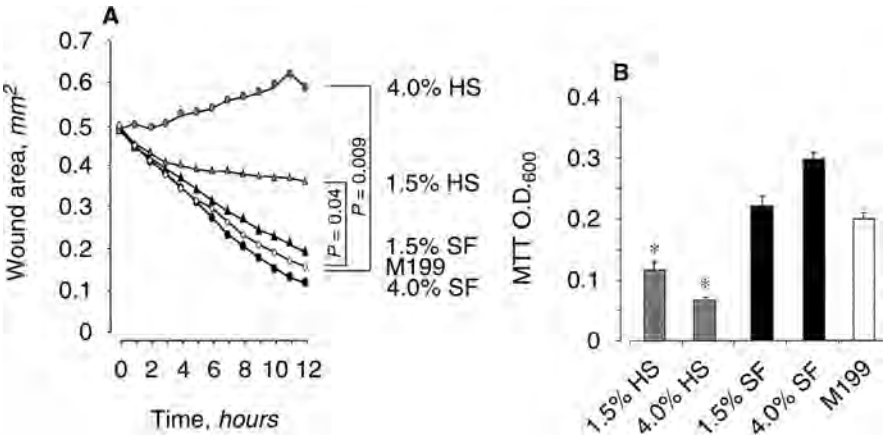

Fig. 2. Time course of remesothelialization (*A*) and cell numbers (*B*) following scratch wounding in human peritoneal mesothelial cells (HPMC) exposed to heat-sterilized (HS) and sterile-filtered (SF) laboratory-prepared dialysis solutions and control medium (M199). Data presented are the mean ($N = 6$ separate experiments performed in quadruplicates) changes in wound areas at the indicated time points in HPMC exposed to 25% vol/vol dilutions of the test and control (M199) solutions. The rate of remesothelialization was significantly retarded in both 1.5% HS and 4.0% HS compared to M199 ($N = 6$; $P = 0.04$ and $P = 0.009$, respectively, Wilcoxon signed ranks test). Remesothelialization rate was unaffected by exposure to sterile filtered solutions. At the conclusion of the experiment (72 hours), cell numbers were assessed by 3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay and were significantly reduced compared to M199 in both 1.5% and 4.0% heat-sterilized solutions ($P < 0.05$ for both) (B).

(Fig. 3A). The complete retardation of remesothelialization in Gambrosol® was in each case associated with significant reductions in HPMC viability and disruption of the monolayer as assessed by total ATP content (Fig. 3B) and direct visualization (Fig. 3C). Cell viability and remesothelialization remained unaffected in Gambrosol-trio®, regardless of glucose concentration (Fig. 3B).

In order to demonstrate the specificity of the observed effects on remesothelialization in subsequent experiments, wounded HPMC were exposed to decreasing dilutions of commercial peritoneal dialysis fluids (10% to 80% vol/vol) in M199. Regardless of dilution or glucose concentration, exposure of wounded HPMC to Gambrosol-trio® was not associated with significant alterations in the rate of remesothelialization [1.5% Gambrosol-trio® (80% vol/vol) 22.7 ± 2.7 μm^2/hour vs. M199 29.7 ± 2.3 μm^2/hour ($N = 5$, P = NS)] or reductions in cell viability (data not shown) (Fig. 4C and D). In contrast, exposure of wounded HPMC to Gambrosol® was associated with a dose-dependent retardation of remesothelialization (Fig. 4A and B). At dilution of 1.5% and 4.0% solutions above 10%vol/vol, the remesothelialization process was significantly retarded and was prevented at dilutions above 25% vol/vol (11.7 ± 1.2 μm^2/hour) and 20% vol/vol (−7.0 ± 6 μm^2/hour) for Gambrosol® 1.5% and 4.0%, respectively ($N = 5$, $P < 0.05$ for all) (Fig. 4 A and B). Concentrations above 50% vol/vol and 30% vol/vol for 1.5% and 4.0% Gambrosol®, respectively, were associated with reductions in HPMC viability (data not shown).

Impact of D-glucose on remesothelialization

The above data were suggestive of little impact of D-glucose per se on the process of remesothelialization. To confirm these observations, a series of D-glucose solutions (5 to 80 mmol/L) were prepared in M199/HEPES at pH 7.4. Exposure of wounded HPMC to solutions containing increasing concentrations of glucose was not associated with significant retardation of remesothelialization, regardless of glucose concentration (M199, 46.1 ± 6.8 μm^2/hour; 80 mmol/L D-glucose, 37.5 ± 5.58 μm^2/hour) (Fig. 5A and C). HPMC viability was not significantly affected by exposure to glucose concentrations up to 80 mmol/L (Fig. 5B) nor was there any difference in cell number as assessed by MTT staining (data not shown).

Remesothelialization in the presence of specific GDP

To identify which GDP species were responsible for specific effects in retarding remesothelialization, wounded HPMC were exposed to individual GDP species at concentrations five times that detected in commercial 1.5% peritoneal dialysis fluids. Attenuation of remesothelialization was only observed in wounded HPMC exposed to formaldehyde and 3,4-DGE ($N = 6$, $P = 0.005$ for both), whereas the other GDP species had no effect on the healing process (Fig. 6A).

Remesothelialization in the presence of mixtures of GDP

To further confirm these observations on the specific effects of formaldehyde and 3,4-DGE and to examine if additive effects were evident, a subsequent series of experiments compared the responses of these GDP species singly (1× concentration, 5 μmol/L and 10 μmol/L formaldehyde and 3,4-DGE, respectively), with a combination of each at these same concentrations and a cocktail of all of the GDP (glyoxal, methylglyoxal, 5-HMF, acetaldehyde, formaldehyde, 3,4-DGE, and 3-DG) at the concentrations found in 1.5% heat-sterilized peritoneal dialysis fluids (1× mix). Both 1× formaldehyde

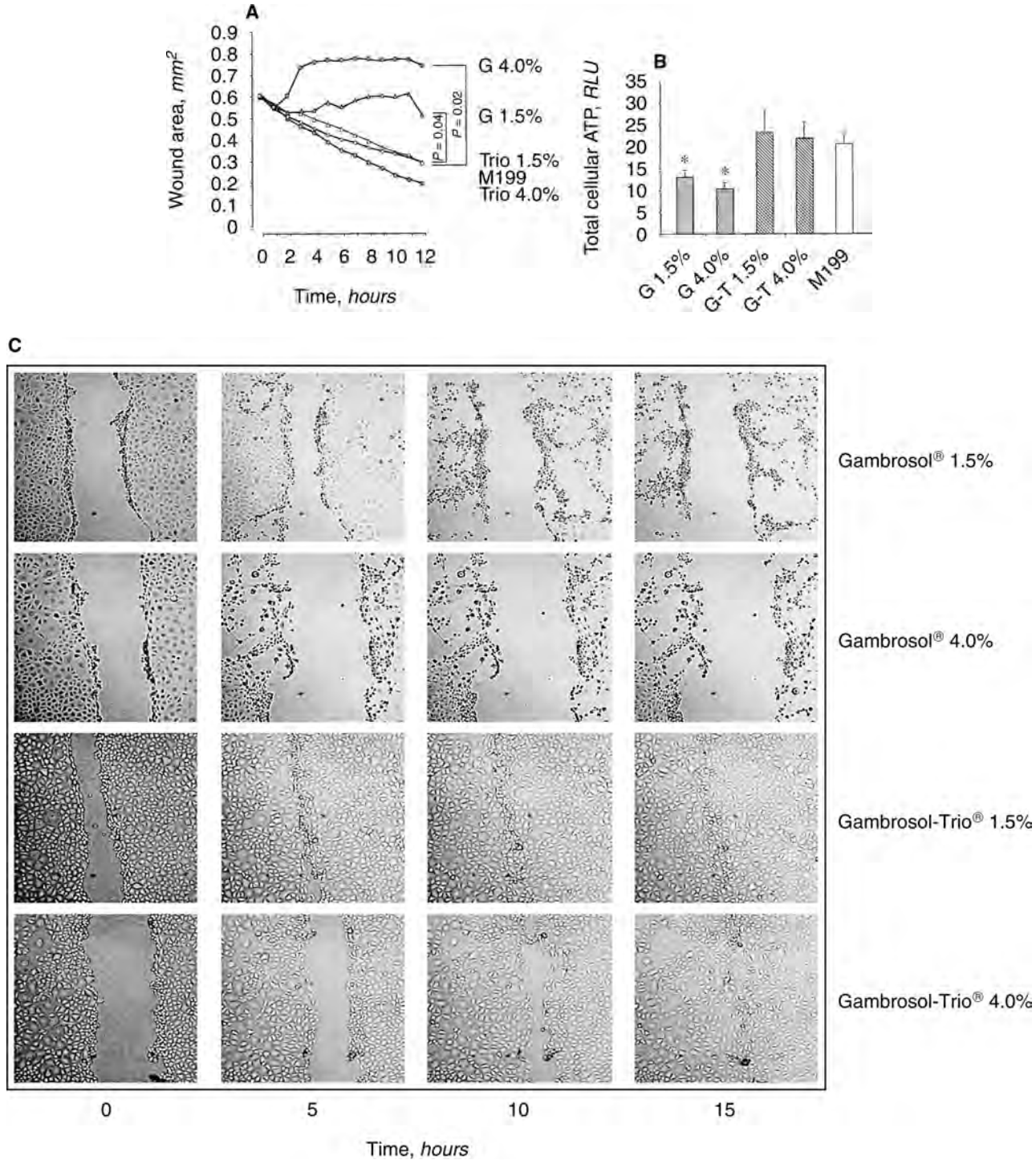

Fig. 3. Time course of remesothelialization (*A*), viability (*B*), and time-lapse photography (*C*) following scratch wounding in human peritoneal mesothelial cells (HPMC) exposed to Gambrosol® (G) (1.5% and 4.0%), Gambrosol-trio® (Trio) (1.5% and 4.0%) or to control medium (M199). Data are the mean ($N = 6$ separate experiments performed in quadruplicate) wound areas at the indicated time points in HPMC exposed to 25% vol/vol dilutions of the test solutions or to M199. The rate of remesothelialization was significantly retarded in both Gambrosol® (1.5% and 4.0%) solutions compared to M199 ($N = 6$; $P = 0.04$ and $P = 0.02$, respectively, Wilcoxon signed ranks test. Remesothelialization rate was unaffected by exposure to Gambrosol-trio® (1.5% and 4.0%). At the conclusion of the experiment (72 hours), cell viability was assessed by measuring total cellular adenosine tri phosphate (ATP), which was significantly reduced (compared to M199) in both Gambrosol® (1.5% and 4.0%) solutions ($N = 6$; $P < 0.05$ for both) (B). Time-lapse photographs demonstrate the failure of wound closure in HPMC exposed to Gambrosol® (1.5% and 4.0%) but complete closure by between 10 and 15 hours in HPMC exposed to Gambrosol-trio® (1.5% and 4.0%) solutions (representative pictures are presented).

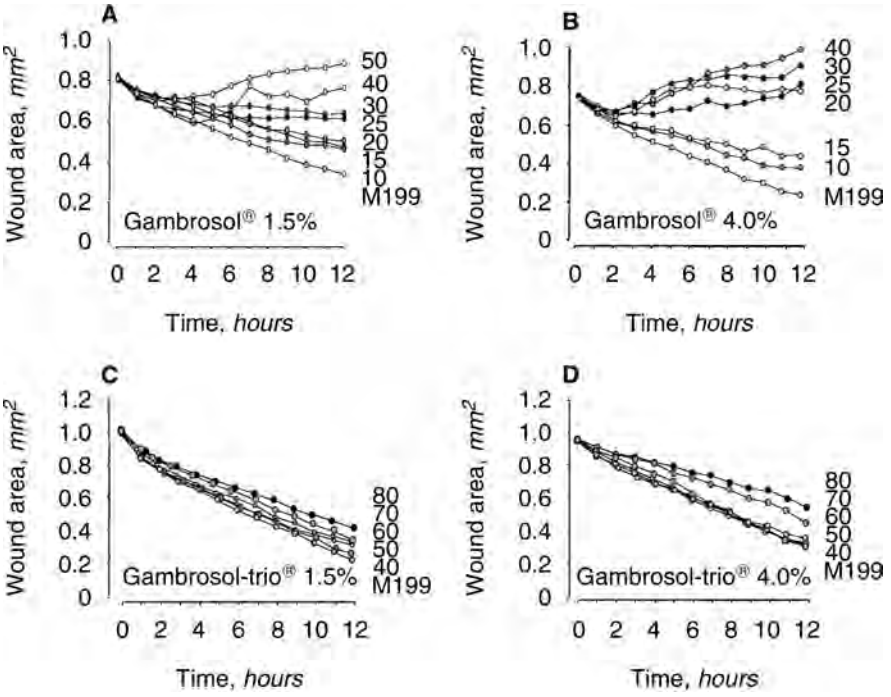

Fig. 4. Time course of remesothelialization following scratch wounding in human peritoneal mesothelial cells (HPMC) exposed to increasing dilutions (10% to 50% vol/vol) of Gambrosol® (1.5% and 4.0%) (*A* and *B*) and (40% to 80% vol/vol) Gambrosol-trio® (1.5% and 4.0%) (*C* and *D*) or to M199. Data are the mean wound areas at the indicated time points in HPMC exposed to test solutions or to M199. The rate of remesothelialization was significantly retarded in all Gambrosol® (1.5% and 4.0%) solutions above 10% vol/vol compared to M199 ($N = 6$; $P < 0.05$ for all Wilcoxon signed ranks test). The rate of remesothelialization was not retarded at any of the dilutions of Gambrosol-trio® (1.5% and 4.0%).

and 3,4-DGE significantly retarded remesothelialization (16 ± 3.6 μm²/hour and 21 ± 4.2 μm²/hour vs. 34 ± 3.2 μm²/hour for M199) ($N = 5$, $P = 0.04$ for both). The combination of 1× formaldehyde and 1× 3,4-DGE resulted in a further reduction of the rate of closure (11.4 ± 1.75 μm²/hour) ($N = 5$, $P < 0.05$ vs. M199) that was similar to that observed in the complete GDP cocktail (10 ± 4 μm²/hour) ($N = 5$, $P = 0.02$ for both vs. M199) (Fig. 6B). Cell viability was not reduced following exposure to 5× glyoxal, methylglyoxal, 3-DG, 5-HMF, or acetaldehyde to the mixtures of formaldehyde and 3,4-DGE (1×) or to the mixture of all GDP (1× mix) (data not shown).

To confirm these observations, wounded HPMC were exposed to increasing concentrations of formaldehyde and 3,4-DGE. In these experiments, both 3,4-DGE and formaldehyde dose-dependently attenuated the remesothelialization process (Fig. 7A and C). Cell viability was only significantly reduced at concentrations of 3,4-DGE above 30 μmol/L (3×) and formaldehyde above 15 μmol/L (3×) (Fig. 7B and C).

DISCUSSION

In the current study, we have utilized a well-characterized model of remesothelialization to examine the impact of GDP-containing solutions on the process of mesothelial cell regeneration following injury [8, 9]. These experiments have specifically examined the effect of GDP in the absence of effects of pH, loss of cell viability, or the confounding effects of growth factor–rich FCS. The data indicate that the endogenous process of mesothelial cell repair is specifically retarded by GDP species and appears to be independent of specific effects of D-glucose at the concentrations present in low glucose peritoneal dialysis fluids.

Interest in the importance of GDP as potentially bioincompatible components of commercial peritoneal dialysis fluids stems from the early observation that infusion pain in patients on peritoneal dialysis was increased when fluids had been stored for a greater length of time [43]. The high concentrations of glucose in peritoneal dialysis fluids decompose spontaneously during storage, but this decomposition process is accelerated by heat sterilization and results in the formation of de novo degradation products of glucose [44, 45]. Subsequent investigations using a range of in vitro test systems have not only identified many of the specific GDP species but also identified that, either in combination (as occurs in heat-sterilized peritoneal dialysis fluids) or singly, these reactive aldehydes and carbonyls have significant capacity to modulate cell viability and function both in vitro and in vivo [19, 20, 22, 23, 46, 47].

Although initial experiments characterizing the global impact of GDP concentrated predominantly on the mouse L929 fibroblast system [19], more recently the mesothelium, the primary target of peritoneal dialysis fluid effects in the peritoneal cavity, has become the test system of choice. Interestingly, these cells appear to be even more susceptible to GDP-mediated effects that the

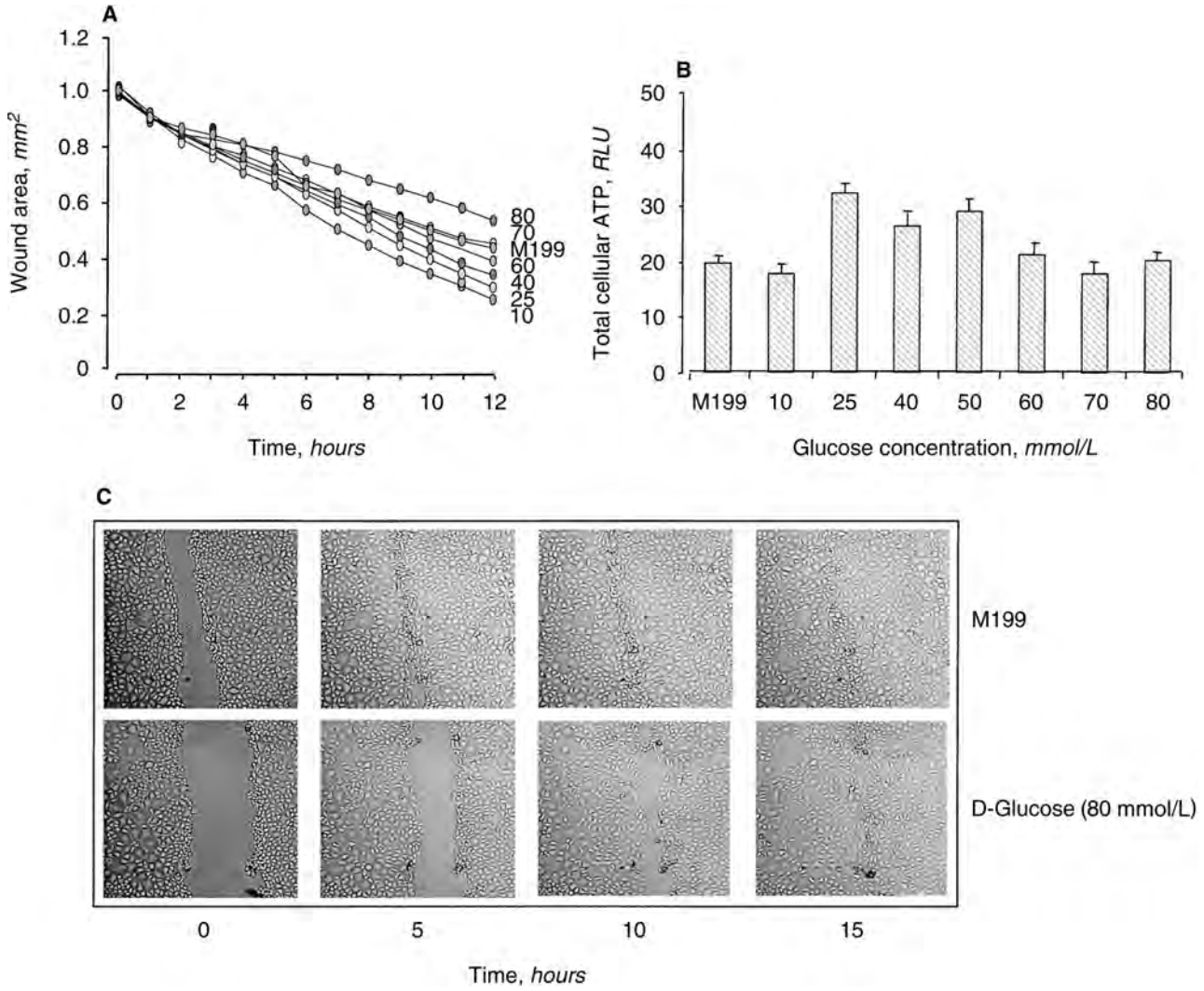

Fig. 5. Time course of remesothelialization (*A*), viability (*B*), and time-lapse photography (*C*) following scratch wounding in human peritoneal mesothelial cells (HPMC) exposed to increasing concentrations (10 to 80 mmol/L) of D-glucose or to control medium (M199). Data are the mean ($N = 6$ separate experiments performed in quadruplicate) wound areas at the indicated time points. Neither the rate of remesothelialization (A) nor cell viability [adenosine triphosphate (ATP) assay] (B) was significantly affected by exposure to D-glucose. Representative time-lapse video images (C) of the remesothelialization process in D-glucose (80 mmol/L) and M199.

transformed L929 cell line [23]. While these initial observations were important in defining the increased sensitivity of mesothelial cells to combined and individual GDP-mediated effects, in general, for significant modulation of biologic effects (cytokine and growth factor release and cell viability) to occur, individual GDP needed to be used at concentrations well above those found in high glucose peritoneal dialysis fluids [21–23, 48]. Although these results may be partly explained by the volatility and reactivity of GDP, resulting in lower effective concentrations than those originally added to the test system, they do not mimic the significant modulation of cellular function attributed to the presence of GDP in heat-sterilized peritoneal dialysis fluids [19]. Nevertheless, these data provided pointers to the differences in individual GDP biologic reactivity [23].

In more relevant experiments, Witowski et al [20] have chronically exposed mesothelial cell monolayers to a mixture of GDP at the maximum concentrations present in 4.0% glucose solutions. These experiments, although unequivocally showing specific GDP-mediated effects on cell function [cell proliferation and interleukin-6 (IL-6) synthesis], were associated with time-dependent loss of cell viability (approximately 75% reduction) and loss of monolayer integrity (although the cells remaining after 36 days showed normal cell proliferation when converted to new GDP free-serum–containing medium).

In the current study, we have attempted to identify spe-

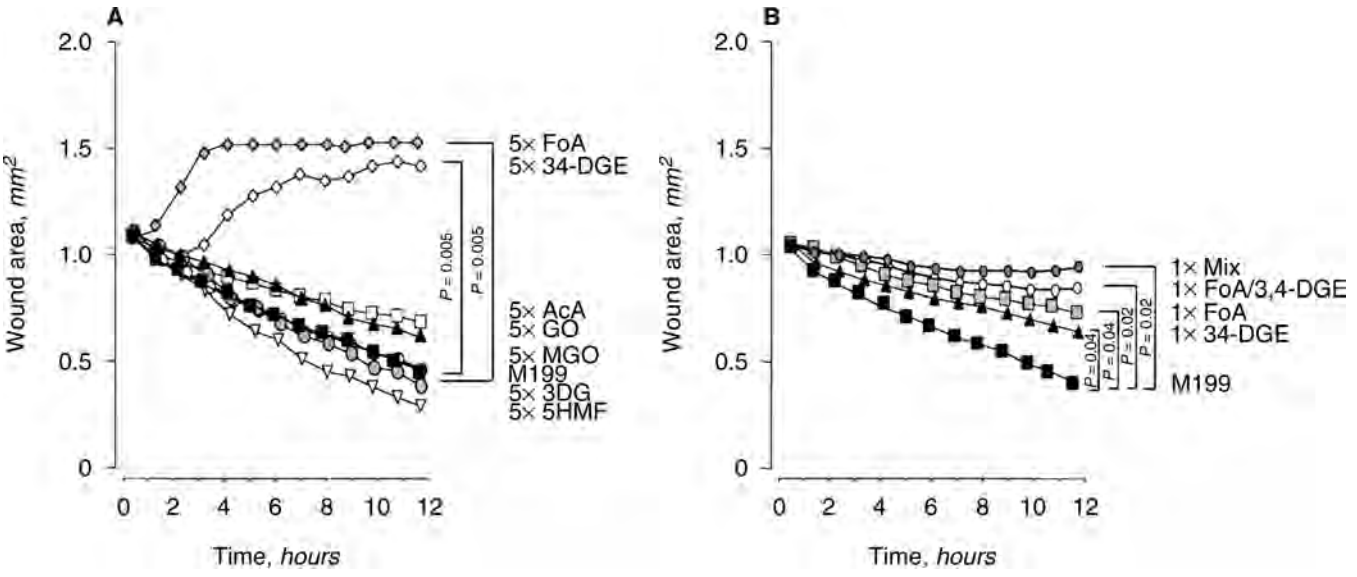

Fig. 6. Time course of remesothelialization following scratch wounding in human peritoneal mesothelial cells (HPMC) exposed to individual glucose degradation products (GDP) [formaldehyde (FoA), 3,4-di-deoxyglucosone-3-ene (3,4-DGE), acetaldehyde (AcA), glyoxal (GO), methylglyoxal (MGO), 5-hydroxy methylfufural (5-HMF), and 3-deoxyglucosone (3-DG)] at five times the concentrations present in 1.5% glucose peritoneal dialysis fluids (*A*) or to either a mixture of FoA, 3,4-DGE, GO, MGO, 5-HMF, 3-DG FoA, 3,4-DGE, and AcA (1× mix) at the concentrations present in 1.5% heat-sterilized peritoneal dialysis fluids or singly to FoA and 3,4-DGE at the concentrations present in 1.5% heat-sterilized peritoneal dialysis fluids or to a combination of FoA and 3,4-DGE (*B*). Data are the mean ($N = 5$ separate experiments) wound areas at the indicated time points. The rate of remesothelialization was significantly retarded in 5× and 1× FoA, 1× 3,4-DGE, the combination of FoA and 3,4-DGE and the 1× mix compared to M199 ($N = 5$; P values as indicated, Wilcoxon signed ranks test.

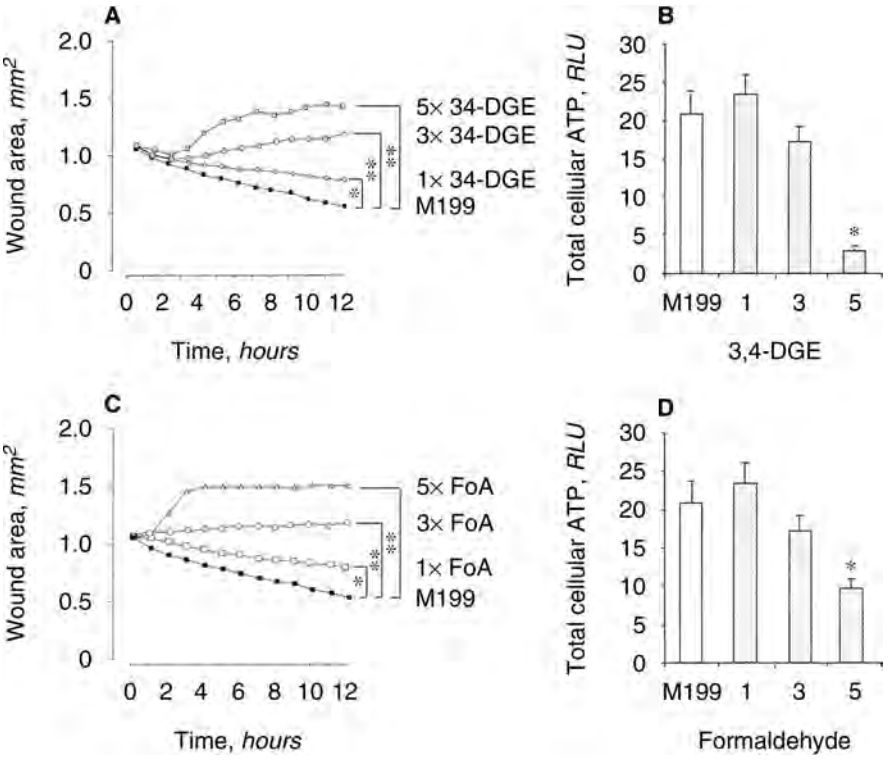

Fig. 7. Time course of remesothelialization (*A* and *C*) and viability (*B* and *D*) following scratch wounding in human peritoneal mesothelial cells (HPMC) exposed to increasing concentrations (1×-5×) of formaldehyde (FoA) and 3,4-di-deoxyglucosone-3-ene (3,4-DGE). Data are the mean ($N = 5$ separate experiments) wound areas at the indicated time points. The rate of remesothelialization was significantly retarded at all concentrations of FoA (A) and 3,4-DGE (C) compared to M199 ($N = 5$). *$P = 0.05$; **$P = 0.02$ Wilcoxon signed ranks test. Cell viability as assessed by total adenosine triphosphate (ATP) concentration was significantly reduced by both 5× FoA and 3,4-DGE compared to M199 ($N = 5$; $P = 0.04$ for all Wilcoxon signed ranks test).

cific GDP-mediated effects (to include those of the recently described 3,4-DGE) on the process of remesothelialization at concentrations relevant to the majority of peritoneal dialysis exposure (i.e., 1.5% glucose solutions) and in the absence of a significant effect on cell viability. Although initial experiments with heat-sterilized laboratory and commercial solutions (diluted 1:4 following previously published protocols and preliminary viability assessments in monolayer cultures) showed inhibition of remesothelialization, these effects were in wounded cells clearly influenced by loss of mesothelial cell viability. These data identify once again the extreme sensitivity of cultured mesothelial cells to dialysis fluid and GDP-mediated effects but also that extrapolation from monolayer to wounded systems needs to be interpreted with caution as the latter appears to be more sensitive to the cytotoxic effects of peritoneal dialysis fluids. The magnitude of effect in these dialysis fluid experiments, compared to the individual and combined GDP effects (see later), clearly identifies, however, that it is the combination of GDP in commercial solutions that mediates the most significant biologic effects and/or that other dialysis solution components or combinations thereof contribute to the observed modulation of cell behavior. In this respect, both individual and combined effects of other dialysis fluid components, including osmolality, glucose, and lactate concentration have been observed in several cell systems [12–15, 49]. To date, however, whether these synergize with GDP in modulating cell function has not been studied. In the majority of previous studies aimed at identifying specific GDP effects, individual or combined levels either at or above those in (3.86% to 4.5%) have been used. Interestingly, the effects observed in our remesothelialization system with conventional heat-sterilized solutions were manifested at GDP concentrations below those present in low glucose peritoneal dialysis fluids (since both 1.5% and 4.0% solutions were diluted 1:4 for these experiments). These observations confirm both the sensitivity of the test system (see earlier) and the fact that the combinations of GDP (many of which remain to be identified) present even in conventional low glucose peritoneal dialysis fluids are likely to have relevant biologic effects within the peritoneal cavity in vivo. That exposure of the peritoneal cavity to such low concentrations of GDP is more relevant to the in vivo situation is emphasized by recently published data documenting their rapid disappearance or sequestration from the dialyzed peritoneal cavity and their contribution to systemic AGE formation [50].

Cumulative exposure to dialysis fluid glucose has previously been implicated as a major causative factor in both functional and structural changes in the dialyzed peritoneum [3, 51]. Although data on mesothelial cell proliferation and activation has implicated D-glucose in mediating cell activation mediated by protein kinase C (PKC) and polyol pathway activation [12–15, 52], the data from the current study suggest that glucose per se has little direct effect in modulating the remesothelialization process. In our remesothelialization experiments, D-glucose at concentrations relevant to 1.5% (up to 80 mmol/L) peritoneal dialysis fluids had no significant effect on remesothelialization or cell viability. These data suggest that the effects observed following exposure of mesothelial cells to peritoneal dialysis fluids are GDP specific. Given that previous studies have identified that increased cell migration and cell proliferation control this process, these data suggest that GDP may have specific effects on both these processes [8–11]. In this respect, GDP exposure has been previously shown to have direct effects on L929 and mesothelial cell proliferation [19, 20, 23].

That significant effects attributable to GDP content in the absence of significant effects on cell viability were further demonstrated in experiments using serial dilutions of commercial high and low GDP solutions in which significant effects were only seen with conventional heat-sterilized peritoneal dialysis fluids (Gambrosol®), whereas low GDP solutions (Gambrosol-trio®) did not retard the remesothelialization process. These data parallel recent observations using intravital microscopy of mesenteric vessels in which the retardation of leukocyte rolling and margination as a result of exposure to GDP containing peritoneal dialysis fluid was significantly reduced with low GDP solutions [47].

Based on the hypothesis that individual GDP species would have less effect than combined exposure (c.f., the dialysis solution experiments), initial experiments exposed wounded mesothelial cells to concentrations of individual GDP at five times the concentration present in low glucose solutions. The majority of the GDP tested were without effect on remesothelialization or cell viability. While it is well established that 5-HMF (although used as a marker of overall glucose degradation) has little biologic effect in test systems, other GDP species, including acetaldehyde, glyoxal, and methylglyoxal, have been shown to modulate mesothelial cell and endothelial cell function and viability [20, 22, 23]. In these experiments, however, individual or combined GDP exposure protocols used much higher concentrations than in the current study to obtain significant effects, that in many cases were associated with significant loss of cell viability.

Only two of the GDP species tested, formaldehyde and the recently described dicarbonyl 3,4-DGE, had a significant effect on the process, although this was clearly the result of direct cell cytotoxicity in these initial experiments [37, 53]. Subsequent experiments demonstrated that the effects of formaldehyde and 3,4-DGE on the remesothelialization were dose-dependent, with significant retardation of the process without effects on cell viability occurring at concentrations relevant to those

present in 1.5% conventional peritoneal dialysis fluids. These data concur with previous studies in HPMC monolayer cultures that have suggested that formaldehyde is one of the most toxic GDP species [23]. Data on the biologic actions of 3,4-DGE have only recently emerged. This compound, originally isolated from *Sargassum jellmanianum* (a brown seaweed), has been shown to have effects (at concentrations relevant to those present in peritoneal dialysis fluids, 9.1 to 18.3 μmol/L) on macrophage and T-cell proliferation, B-cell antibody production, and more recently to be the most biologically active GDP in the L929 fibroblast proliferation system [53]. The precise mechanisms by which these two GDP species modulate mesothelial cell behavior in the absence of effects on cell viability is not known. It is tempting to speculate, however, that these reactive intermediates in some way modulate the processes of migration and proliferation important in the remesothelialization process [8–11]. These types of effect may be particularly relevant to 3,4-DGE that has been shown previously to modulate macrophage and T-cell proliferation [53]. In addition, the effects on mesothelial cell function may be secondary to AGE formation, known to be accelerated by the presence of GDP [54]. Interestingly, the concentration of 3,4-DGE appears to increase with storage and is an intermediate in the Maillard reaction [53]. Although glucose has the ability to bind nonenzymatically to free amino groups on proteins, resulting in the formation of AGE, it is now well established that glycation mediated directly by GDP may be of equal importance in AGE formation [24, 25, 55]. AGE have been detected in the mesothelium, submesothelial stroma, and vascular walls of peritoneal dialysis patients and correlate with histopathologic changes in the dialyzed peritoneal membrane [27, 28]. Recently, AGE adducts have been implicated indirectly in mesothelial cell and endothelial cell activation and the induction of vascular endothelial growth factor (VEGF) [26]. Taken together, these data add to the spectrum of detrimental activity of GDP in the peritoneal cavity.

The combination of GDP do, however, appear to be important in modulating mesothelial cell function. In the current experiments, GDP, when applied in combinations (as the cocktail found in 1.5% peritoneal dialysis fluids or following addition of formaldehyde and 3,4-DGE), achieved a greater degree of inhibition of remesothelialization than when applied individually. These data concur with previous observations that the maximum effect in any of the biologic assays used to test GDP activity is always mediated by conventional heat-sterilized peritoneal dialysis fluids, emphasizing that the interplay between solution components and GDP species contributes to the overall effects in modulating cell function.

CONCLUSION

The present study has demonstrated that concentrations of GDP relevant to the majority of dialysis dwells significantly modulate remesothelialization by a process that does not necessarily involve a reduction in cell viability. These effects on remesothelialization appear to be mediated primarily by the combination of 3,4-DGE and formaldehyde. Although the mechanisms by which these effects occur are unknown, the dependence of the remesothelialization process on cell migration and cell proliferation suggests that these may be one of the primary targets of GDP action. Considerable clinical interest has been generated in the use of more biocompatible peritoneal dialysis fluids since data from short-term clinical trials has demonstrated that conversion of patients from standard heat-sterilized acidic lactate-buffered peritoneal dialysis fluids containing glucose to potentially more "biocompatible solutions" of neutral or physiologic pH and containing reduced GDP levels have been associated with positive impact on ex vivo cell function, evidence of increased mesothelial cell preservation [29, 31, 33, 34], and recent data on reduced systemic AGE formation [50]. The current observations suggest that solution GDP content might contribute to retarding normal mesothelial turnover in long-term peritoneal dialysis. Recent developments in solution design have concentrated on the overall reduction in GDP profile, the current data suggest that targeting the reduction of 3,4-DGE and formaldehyde in particular may best achieve the goal of mesothelial cell and membrane preservation in long-term peritoneal dialysis.

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