Daily short exposure of cultured mesothelial cells to lactated, high-glucose, low-pH peritoneal dialysis fluid induces a low-profile regenerative steady state

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Abstract This study was designed to evaluate cytotoxic effects and influence upon cell growth of cultured mesothelial cells exposed to modified 4.25% Dianeal dialysate fluid (M-199 in Dianeal solution, glucose 4.25 g, pH 5.2) (Expr. group), 60 min a day for a total period of follow-up of 13 consecutive days, compared with that observed in a control group (C).

Beginning on day 7, the cell counts in group C were significantly higher than those observed at zero time ($P<0.05$). Cell counts in the experimental group showed no significant differences between the first day of culture and each one of the 13 consecutive days of follow-up.

Thymidine incorporation into DNA observed on the first day in C, was significantly higher ($P<0.01$) beginning on the 10th day. Values observed in the experimental group were low during the whole period of follow-up.

Repeated exposure of the mesothelium to 40 mMol/l lactate and high glucose concentrations induced severe cell injury and death, decreased cell growth and, consequently, a reduced rate of regeneration which is extended as long as the repeated exposure is maintained.

Key words: biocompatibility of dialysis solutions; mesothelial cells culture

Introduction

Peritoneal dialysis involves repeated and long-standing exposure of the peritoneum to acidic, potassium free, high glucose, and hyperosmolar lactate-buffered solutions, containing variable amounts of products derived from the non-enzymatic degradation of glucose [1]. Consequently, as shown in a previous study done in patients on PD, the exposed mesothelium is repeatedly injured, and this is associated with continuous regeneration [2]. The main ultrastructural changes detected are disappearance of microvilli, hyperplasia of rough endoplasmic reticulum, disruption of tight junctions and decreased numbers of micropinocytotic vesicles in the cytoplasm [3,4].

The more commonly observed functional changes demonstrated in blood cells exposed to commercially available peritoneal dialysis fluids are suppression of chemiluminescence, phagocytosis and bacterial killing of PMN [5-7], macrophages and monocytes [8,9], and reduced leukotriene and TNF release from PMS [10,11]. Evidence obtained from cultured mesothelial cells exposed to low pH lactated dialysis fluid, for periods of time ranging from 6 to 72 h [12-15], showed signs of more or less severe cytotoxicity, together with substantial inhibition of cell growth. In vitro studies using extended incubation periods do not represent the continuously changing steady-state characteristic of the in vivo situation. During peritoneal dialysis, pH and potassium levels of the dialysate reach those serum levels, after 40-60 min of instillation [5]. A more recent study [16] showed that human peritoneal mesothelial cells synchronized at the G1/S phase boundary, acutely exposed to lactate-buffered, 1.36% glucose dialysis fluid for 15 min, developed severe injury as evidenced by increased LDH release and reduced ATP levels. Looking for an approach closer to the clinical set-up, we tried to develop an in vitro system using isolated mesothelial cells to reproduce a once-a-day CAPD regimen.

Consequently, this study was designed to evaluate cytotoxic effects and influence upon cell growth of cultured mesothelial cells exposed to modified 4.25% Dianeal dialysate fluid for 60 min a day, for 13 consecutive days.

Subjects and methods

Cell harvesting and primary culture

These were performed according to Sato et al. [17], with a slight modification. Albino rats weighting 160-200 g were...
injected intraperitoneally with 40 ml HBSS without phenol red, and 0.125% trypsin for 30 min. The aspirated fluid was centrifuged at 1500 r.p.m. for 10 min. The pellet was washed thrice with medium M-199 and 20% FCS, and centrifuged again. The cell suspension was plated in 25 cm² tissue culture flasks (Corning Glass Works, Corning, NY 25106). Sixty minutes later the plates were washed with medium, and incubated at 37°C–5% CO₂–in M-199 medium, supplemented with 20% FCS, penicillin G-100 U/ml, streptomycin 100 µg/ml, amphotericin B-0.25 µg/ml, until confluency. Nutrients were added every 3 days.

Identification of cells

Cells were identified as mesothelial, based on their immunohistochemical staining performed with monoclonal antibodies to rat pancytokeratin, cytokeratin 8 vimentin, macrophages (Chemicon, Temecula, CA), and Factor VIII (Biomaker, Israel). These were visualized by FITC goat anti mouse IgG (Gamma) (Zymed Laboratories, Inc.). All cells were positively stained for cytokeratins and vimentin, whereas no factor VIII antigen and monococyte/macrophage antigen was detected.

Growth study

A monolayer of confluent mesothelial cells was harvested with 0.25% trypsin and 0.05% EDTA, resuspended in fresh medium M-199, supplemented with 20% FCS, centrifuged twice at 1500 r.p.m. for 5 min, and seeded into 25-cm² tissue culture plates at a density of 2 × 10⁴/ml fresh medium M-199 containing 1% FCS for 24 h. The reason for using medium with low FCS concentration for a short period of time was to synchronize most cells at the same phase (G1 phase) using the technique of nutritional deprivation [16,18–21]. It is known that the synchrony decays because of the intrinsic variation in cycle times among cells, a biological phenomenon inherent to most cell types. However, numerous investigators [16,18–21], have shown that this method requires that most treated cells are initially at the G1/S boundary.

Cultures were exposed to medium M-199 modified Hanks' salt base in Dian Neal solution, glucose 4.25 g/l, pH 5.2, containing 10% FCS (Biological Industries, Israel) for 60 min. This methodology was designed because preliminary experiments showed that confluent mesothelial cells in culture exposed to 4.25% Dianeeal solution for 30 min were completely detached and washed away. Control plates were exposed to fresh medium, supplemented with 10% FCS and antibiotics. Cell counts were performed once a day for 13 days. Twenty-four hours after each exposure, the medium was aspirated for LDH determination, and plates were washed with PBS, exposed to 0.25% trypsin and 0.05% EDTA for 3 min. at 37°C, verifying by microscopy that all cells were detached from the plate. Then, trypsin activity was stopped by the addition of medium supplemented with 20% FCS. Cell suspensions were repeatedly passed through a fine Pasteur pipette to disrupt clumps.

Aliquots were counted in a haemocytometer. Other aliquots were mixed with 0.4% trypsin blue in PBS, and counted in a haemocytometer. This procedure was performed to obtain a quantitation of the contributions of non-viable cells to total cell numbers in each culture. Analysis of data obtained from cell counts was done using the respective actual number of viable cells observed in each experiment.

DNA synthesis

3H thymidine incorporation into cell DNA was used as an index for cell proliferation and expressed in counts per min. (c.p.m. 10⁴) per plate, as well as counts per number of viable cells. Control and experimental plates were pulsed with 3H thymidine (5 µCi/mmole, Rotem Industries, Israel) by adding 1 µCi/ml medium for 60 min. The medium was then removed and cells were washed three times with PBS. Then 1 N NaOH was added and after 30 min the suspension was collected into scintillation vials. The radioactivity was assayed by counting the NaOH suspension in scintillation cocktail for β emissions in an LKB Rack Beta Counter. Each experimental condition was tested in duplicate eight times, and the mean was taken to represent each individual experiment. Data are presented as absolute counts (c.p.m. 10⁴) per plate, as well as counts (c.p.m. 10⁴) per number of viable cells.

LDH measurements

Activity of lactate dehydrogenase, a cytosolic enzyme of large molecular weight that is released only on severe cellular damage, was measured every 24 h on each experimental and control plates, to provide quantitative evaluation of cell detachment and death. LDH measurements were performed according to Wroblewski and La Due on the medium aspirated from each plate [22]. Actual LDH figures were obtained by subtracting the LDH levels of fresh medium supplemented with 10% FCS. Data are presented as absolute values of LDH, as well as IU/L per number of viable cells per millilitre.

Statistical analysis

Data are presented as mean±SD. As stated in the text, differences between groups were evaluated using the two-tailed Wilcoxon test and the two-tailed Bonferroni method of multiple comparisons against a single control. The level of significance was considered to be P<0.05. Some results were further analysed for α=β=0.05, to define the minimal required sample size.

Results

At the time of plating, over 95% of cells were viable as indicated by trypan blue. Plating efficiency was identical for both, control and experimental groups. Cell counts obtained for each group (control and experimental) on the first day were compared with counts made on the following days by using the two-tailed Bonferroni method (Figure 1). Beginning on the 7th day, cell counts in the control group were significantly higher than those observed at the beginning (P<0.05 on days 7 and 8, P<0.01 on day 9 and P<0.001 on days 10–13, according to the Bonferroni method of multiple comparisons against a single control). However, cell counts of the experimental group showed no significant differences between the first day of culture and each one of the 13 consecutive days of follow-up. When paired data from both groups were compared at each time interval, differences were also statistically significant beginning on the 7th day of follow-up (Figure 1).
Cell counts obtained on the first day in each group (control and experimental) were compared with counts made on the following days by using the two-tailed Bonferroni method. Beginning on the 7th day, values obtained in the control group were significantly higher than those observed on the 1st day (\(P<0.05\) on days 7 and 8, \(P<0.01\) on day 9, and \(P<0.001\) on days 10-13), whereas figures obtained in the experimental group showed no significant differences between the first day of culture and the 13 days of the experimental study. Data obtained from control and experimental groups at each time interval are compared using the paired two-tailed Wilcoxon test (\(n=8\); NS; \(x, P<0.05\); \(xx, P<0.01\); \(xxx, P<0.001\)).

Thymidine incorporation into DNA (Figure 2), observed on the first day in both the experimental and control groups, was compared with counts made on the following days of the whole observation period. In the control group, differences in thymidine incorporation were significant beginning on the 11th day. However, values observed for the experimental group did not significantly change during the whole study period. Longitudinal comparison of thymidine incorporation (c.p.m. \(10^3\)/number of viable cells) (Table 1) showed that for the control group, counts \(10^3\)/number of viable cells reached statistical significance on the 11th experimental day. The lack of significant difference on the 10th day compared with that observed in total counts at the same time interval, derives from the divergence between total counts and those based only on viable cells. Longitudinal comparison of counts \(10^3\)/number of cells in the experimental group confirmed the homogeneity and the absence of significant differences between values observed at each time interval compared with those observed on the 1st day. Indeed, paired comparison of thymidine incorporation/number of viable cells between control and experimental groups at each time interval detected significant differences at the \(P<0.001\) level, beginning on day 11 (Table 1).

LDH levels (Figure 3) observed in each group during the 13 days of the study period were not significantly different from those detected on the 1st day of culture. However, comparison of the control and the experimental groups at each time interval showed that LDH levels were significantly higher \((P<0.001)\) in cultures exposed to the high glucose concentration lactated fluid. (Two-tailed Wilcoxon test. \(n=8\); \(xxx, P<0.001\)).

**Table 1.** 3H Thymidine (c.p.m.) per number of cells. Data are presented as mean \((10^3)\)±SD. Longitudinal comparison for each group was done with the Bonferroni method (\(xxx, P<0.001\)).

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>Experimental</th>
<th>(P) (Paired comparison, Wilcoxon test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.0±6.6</td>
<td>38±20.0</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>55.4±42.0</td>
<td>45.1±21.7</td>
<td>NS</td>
</tr>
<tr>
<td>3</td>
<td>31.5±18.0</td>
<td>23.5±4.2</td>
<td>NS</td>
</tr>
<tr>
<td>4</td>
<td>63.4±46.0</td>
<td>54.1±32.0</td>
<td>NS</td>
</tr>
<tr>
<td>5</td>
<td>15.5±2.1</td>
<td>24.3±10.9</td>
<td>NS</td>
</tr>
<tr>
<td>6</td>
<td>38.9±18.0</td>
<td>48.1±14.0</td>
<td>NS</td>
</tr>
<tr>
<td>7</td>
<td>33.5±17.0</td>
<td>42.5±21.0</td>
<td>NS</td>
</tr>
<tr>
<td>8</td>
<td>41.4±18.1</td>
<td>35.4±12.7</td>
<td>NS</td>
</tr>
<tr>
<td>9</td>
<td>20.6±6.0</td>
<td>30.8±5.6</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>52.7±30.0</td>
<td>35.7±7.7</td>
<td>NS</td>
</tr>
<tr>
<td>11</td>
<td>86.0±28.7xxx</td>
<td>32.8±13.4</td>
<td>&lt;0.001 - for (s=\beta=0.05) (n=6)</td>
</tr>
<tr>
<td>12</td>
<td>150.1±6.9xxx</td>
<td>32.8±7.4</td>
<td>&lt;0.001 - for (s=\beta=0.05) (n=2)</td>
</tr>
<tr>
<td>13</td>
<td>148.0±1.8xxx</td>
<td>47.3±19.1</td>
<td>&lt;0.001 - for (s=\beta=0.05) (n=2)</td>
</tr>
</tbody>
</table>

**Fig. 1.** Cell counts obtained on the first day in each group (control and experimental) were compared with counts made on the following days by using the two-tailed Bonferroni method. Beginning on the 7th day, values obtained in the control group were significantly higher than those observed on the 1st day (\(P<0.05\) on days 7 and 8, \(P<0.01\) on day 9, and \(P<0.001\) on days 10-13), whereas figures obtained in the experimental group showed no significant differences between the first day of culture and the 13 days of the experimental study. Data obtained from control and experimental groups at each time interval are compared using the paired two-tailed Wilcoxon test (\(n=8\); NS; \(x, P<0.05\); \(xx, P<0.01\); \(xxx, P<0.001\)).

**Fig. 2.** 3H thymidine incorporation observed on the 1st day for both groups was compared with counts made on the following days of the whole observation period. In the control group, differences in thymidine incorporation were significant beginning on the tenth day. However, values observed for the experimental group did not significantly change during the whole study period. Longitudinal comparison of thymidine incorporation (c.p.m. \(10^3\)/number of viable cells) (Table 1) showed that for the control group, counts \(10^3\)/number of viable cells reached statistical significance on the 11th experimental day. The lack of significant difference on the 10th day compared with that observed in total counts at the same time interval, derives from the divergence between total counts and those based only on viable cells. Longitudinal comparison of counts \(10^3\)/number of cells in the experimental group confirmed the homogeneity and the absence of significant differences between values observed at each time interval compared with those observed on the 1st day. Indeed, paired comparison of thymidine incorporation/number of viable cells between control and experimental groups at each time interval detected significant differences at the \(P<0.001\) level, beginning on day 11 (Table 1).

**Fig. 3.** The Bonferroni two-tailed test showed that in both groups, LDH levels observed during the 13 days of the study period were not significantly different from those detected on the 1st day of culture. However, comparison of the control and the experimental groups at each time interval showed that LDH levels were significantly higher \((P<0.001)\) in cultures exposed to the high glucose concentration lactated fluid. (Two-tailed Wilcoxon test. \(n=8\); \(xxx, P<0.001\)).
the 13 days of 1 h per day exposure were not significantly different from those detected at the 1st day of plating (Figure 3). However, at each time interval, LDH mean values of the control and the experimental group were significantly higher for cells exposed to the high-glucose, lactated solution. Accordingly, cytotoxic effects of the experimental fluid were evident since the first day of exposure. This evidence was further confirmed by comparing LDH levels/number of viable cells at each time interval (Figure 4).

Discussion

There is a growing number of articles describing cytotoxic effects induced by incubation of peritoneal mesothelial cells in different dialysis solutions for variable periods of time. Most of these experiments, however, used extended periods of incubation which reached 18 and even 72 h of duration [12–15]. It should be noted that a recently published study [16] showed substantial cell injury after acute exposure of cultured mesothelium to 1.36% glucose, lactate-buffered dialysis fluid at pH 5.2 for only 15 min.

The purpose of the present study was to develop an in-vitro experimental model qualified to analyse the influence of high-glucose, high-osmolality, low-pH and lactated fluid upon mesothelial cells exposed to one exchange per day during a 2-week period. This was done in accordance with previous studies performed in our laboratory, applying the in vivo and in situ technique of mesothelial cell imprints to mice, the peritoneal cavity of which was exposed to the same fluid for 15 and 30 consecutive days [23–25]. Using this model, the mesothelial monolayer showed signs of severe cellular distress.

The present investigation revealed that a 60-min repeated exposure to a high-glucose, low-pH, lactated dialysis fluid can induce substantial cytotoxicity, as suggested by the high LDH values. This effect is most probably mainly related to the presence of lactate, which has been shown to be cytotoxic for cultured cells in concentrations substantially lower than those used in clinical peritoneal dialysis [26,27].

It should be noticed that LDH was estimated 24 h after exposure each day. The data described in Figure 3 describe LDH release every 24 h and do not represent cumulative values. Furthermore, analysis of LDH release as a function of viable cells (Figure 4), shows that values of both groups were mostly homogeneous, substantiating an experimental variability well within the range of two standard deviations.

It could be argued that such elevation of LDH release in the experimental group might suggest that most exposed cells were irreversibly injured. In fact, injury covers all types of abnormal metabolic deviations, from the very small and perhaps fully reversible to the major irreversible changes that proceed to death [28]. Indeed it has been shown that exposure of cultured human peritoneal mesothelial cells to a low-glucose, lactated and acidic (pH 5.2) solution for a period of 60 min induced a twofold increase of LDH release during a recovery period of 12 h [16]. This manifestation was coincident with a significant decrease in cellular ATP that reverted, however, to control levels after a recovery period of 4 h. This means that the increased release of LDH does not imply that all exposed cells were irreversibly injured. Indeed our observations of lower but still present thymidine incorporation observed in the experimental group at each time interval point at the presence of living cells able to start S phase.

On the other hand, histochemical studies done on mesothelium exposed in vivo to one exchange a day of high-glucose, lactate-buffered dialysis fluid at pH 5.2, showed that this repeated and extended intervention induced a substantially higher activity of plasmalemmal and cytoplasmic enzymes [24]. This same experimental model also revealed that most exposed mesothelial cells (up to 80%) had a surface area two times the size of that observed in normal controls [23,25]. This, in addition to the observed increased mesothelial cell thickness [29] and the profusion of rough endoplasmic reticulum [30] indicate hypertrophic cells with high enzymatic activity. Therefore it is not surprising that in the in vitro setup, severely injured cells, rich in enzymes, were able to release large amounts of LDH.

In addition it should be noticed that the unnatural D-form of lactate, inhibitor of oxidative phosphorylation [31], makes up to 50% of the total buffer of the solution used in this study. That makes a concentration of this D-isomer several hundred times higher than the normal blood level. There is no doubt, however, that the low pH, unchanged during the whole period of exposure, could have had some additional effect, as showed by Gallimore et al. [9], in observations made after an 18-h incubation time, as well as in those reported by Witowski et al. [16], after a 15 min.
incubation. However, cytotoxicity and signs of cell injury were also associated with the same solution at pH 7.3, but with longer periods of incubation [16]. The increase in cell mortality induced by the experimental fluid was associated with lower cell growth. Inhibition of mitotic rate has been observed in endothelial cells exposed to high glucose concentration [32, 33] and appears to be related more to a direct effect of glucose itself than to hyperosmolarity. In this context, observations made by Breborowicz et al. [14] in cultured mesothelial cells showed that equimolar concentrations of mannitol to those of glucose had some detrimental effect upon mesothelial cell growth. This effect was significantly lower than that observed with glucose at every tested concentration. Furthermore the effect of glucose was correlated with its concentration. So far the association of high glucose concentration and lactate appear to have severe detrimental effects upon mesothelial viability and growth.

Cytotoxicity and decreased cell proliferation induced by the experimental solution did not significantly change during the whole observation period which covered a substantial part of the cells’ life span in culture. Consequently it appears that in the stable microenvironment of the in vitro set-up, once-a-day, 60 minute exposure to Dianecol M-199 was enough to prevent the mesothelial cells from recovering their cell numbers by increasing their proliferation rate. Therefore the exposed cells reached and maintained a new low-proliferative steady state, caused by the repeated injury which was partially compensated by means of their inhibited proliferative capabilities.

It is evident that these observations cannot be entirely applied to the in-vivo situation, mainly because of the constancy of the microenvironment characteristic of the in vitro set-up and the long 60 min exposure period. However, on the other hand this study suggests that in vitro repeated exposure of the mesothelium to commercially available solutions with low-pH, 40 mMol/1 lactate and high glucose concentrations induce a different behaviour of cells. Namely, severe cell injury and death, decreased cell growth, and consequently reduced rate of regeneration which is most probably extended as long as the repeated exposure is maintained. This situation will eventually affect both the role of mesothelium in the control of inflammation [16], and the active intervention of the monolayer in the transperitoneal transfer of macromolecular proteins [34, 35].

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