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Original Article

Inhibition of NF-kappaB with Dehydroxymethylepoxyquinomicin modifies the function of human peritoneal mesothelial cells

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Abstract: Peritoneal mesothelial cells exposed to bioincompatible dialysis fluids contribute to damage of the peritoneum during chronic dialysis. Inflammatory response triggered in the mesothelium leading to neovascularization and fibrosis plays an important role in that process. We studied the effects of Dehydroxymethyepoxyquinmicin (DHMEQ)-an NF-κB inhibitor on function of human peritoneal mesothelial cells (HPMC) in *in vitro* culture. DHMEQ studied in concentrations of 1-10 μg/ml was not toxic to HPMC. Synthesis of IL-6, MCP-1 and hyaluronan in unstimulated and stimulated with interleukin-1 (100 pg/ml) HPMC was inhibited in the presence of DHMEQ and the effect was proportional to the dose of the drug. DHMEQ (10 μg/ml) reduced in unstimulated HPMC synthesis of IL-6 (-55%), MCP-1 (-58%) and hyaluronan (-41%). Respective values for stimulated HMPC were: -63% for IL-6, -57% for MCP-1 and -67% for hyaluronan. The observed effects were due to the suppression of the expression of genes responsible for the synthesis of these molecules. DHMEQ modified the effects of the effluent dialysates from CAPD patients on the function of HMPC. Dialysate induced accelerated growth of these cells, and synthesis of collagen was inhibited in the presence of DHMEQ 10 μg/ml, by 69% and 40%, respectively. The results of our study show that DHMEQ effectively reduces inflammatory response in HMPC and prevents excessive dialysate induced proliferation and collagen synthesis in these cells. All of these effects may be beneficial during chronic peritoneal dialysis and prevents progressive dialysis-induced damage to the peritoneum.

Keywords: Peritoneal mesothelium, NF-κB inhibition, inflammation, collagen, peritoneal dialysis

Introduction

Peritoneal dialysis is the procedure which is used in end stage renal failure patients as renal replacement therapy. Intraperitoneal instillation of the dialysis fluid allows for the diffusion of toxic metabolites from the blood, across the peritoneum, into the dialysate. However it is well known that implantation of a peritoneal catheter into the abdominal cavity or single intraperitoneal infusion of the fluid result in induction of the inflammatory response [1, 2]. Additionally that effect is enhanced by the fact that peritoneal dialysis fluids have low biocompatibility due to their pH, electrolyte composition, hyperosmolality, high glucose and glucose degradation products content [3]. The relatively short viability of the peritoneum as the dialysis membrane is the consequence of peritoneal mesothelium damage, overgrowth of the connective tissue and neovascularization [4]. Depending on which effect is stronger, these changes may lead to the formation of the hyperpermeable membrane causing ultrafiltration failure or the hypopermeable membrane which results in reduced elimination of toxins from the bloodstream [5].

In physiological conditions mesothelial cells lining the peritoneal cavity play an important role in the regulation of intraperitoneal homeostasis. They produce proteins regulating the processes of clotting and fibrinolysis [6, 7], adhesion proteins [8], hyaluronan [9] and surfactant [10] which determine their interactions with other cells. Mesothelium produces chemokines

[11], cytokines [12] and proteins of the extracellular matrix such as collagen, elastin and fibronectin [13]. In conditions of peritoneal dialysis, chronic exposure of the mesothelium to bioincompatible dialysis solutions induces the inflammatory response in these cells which may initiate damage to the peritoneum. Some of the observed effects may be mediated via the change in NF-κB factor activity. However the effect of various components of the dialysis fluids on NF-κB activity is not uniform.

Matsuo et al found that exposure of rat peritoneal mesothelial cells to hyperosmolar medium containing high concentrations of glucose or mannitol caused a protein kinase C dependent activation of NF-κB which resulted in increased synthesis of MCP-1 [14]. On the other hand, other researchers described the suppressing effect of acidic and hyperosmotic dialysis fluids on NF-κB activity in mesothelial cells [15]. In the *in vitro* experiments low pH was found to be the main factor responsible for the inhibition of NF-κB activity in mesothelial cells and NF-κB dependent MCP-1 induction [16]. Other compounds present in vast amounts in CAPD patients, glycated proteins strongly stimulate NF-κB in human mesothelial cells with subsequent increased release of inflammatory mediators such as TNF-α, IL-1β, and IL-6 or enhanced activity of the enzymes cyclooxygenase-2 and inducible nitric oxide synthase [17]. The effect of glycated proteins is dependent on the age of the mesothelial cells donor [18]. The increased activity of NF-κB in mesothelial cells cultured *in vitro* in medium with high glucose content results in the increased synthesis of such proteins as fibronectin, collagen 1 or Plasminogen Activation Inhibitor-1 [19]. The epithelial-to-mesenchymal transition of peritoneal mesothelial cells, which is a common disorder in patients on chronic peritoneal dialysis, also depends on NF-κB activation [20].

Control of NF-κB activity in mesothelial cells during peritoneal dialysis may be beneficial and prevent long term deterioration of the peritoneal structure and dysfunction as the dialysis membrane [14, 16, 17, 19]. Dehydroxymethyepoxyquinmicin (DHMEQ), which is a derivative of a weak antibiotic, Epoxyquinomicin C, inhibits its NF-κB activity [21]. DHMEQ suppresses NF-κB activity both in *in vitro* conditions [22, 23] and *in vivo* [24, 25]. In the majority of the experimental *in vivo* studies DHMEQ was administered intraperitoneally. However it was impossible to detect an active concentration of that compound in the blood [26]. It is therefore possible that DHMEQ, after intraperitoneal delivery, is quickly absorbed by the peritoneal immunocompetent cells. We also supposed that mesothelial cells lining the peritoneal cavity may be the target of DHMEQ activity. We present results from *in vitro* experiments on human peritoneal mesothelial cells in which the effects of DHMEQ on these cells was evaluated.

Material and methods

Experiments were performed on primary cultures of human peritoneal mesothelial cells (HPMC). Effluent dialysates used during the experiments were collected from patients treated with continuous ambulatory peritoneal dialysis (CAPD). The study was approved by the bioethics committee at the University of Medical Sciences in Poznan.

Cell culture

HPMC were isolated from pieces of omentum removed during abdominal surgery, by enzymatic digestion following methods used in our laboratory [27]. All cultures were established from healthy individuals with no evidence of uremia, peritonitis, diabetes or peritoneal malignancy. The age of the donors ranged from 32 to 60 years old. Cells were identified as mesothelial by their typical morphology as well as positive staining for Wt-1 and HBME-1 antigens. HPMC were propagated in M199 medium with L-glutamine (2 mmol/L), penicillin (100 U/ml), streptomycin (100 g/ml) and 10% foetal calf serum-FCS (GIBCO, Invitrogen Life Technologies, Paisley UK) at 37°C in 5% CO_2 atmosphere. Cells from the 1st-2nd passage were used in the experiments.

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) and cell culture plastics were obtained from Nunc (Roskilde, Denmark). Dehydroxymethylepoxyquinomicin (DHMEQ) was synthesized in the Aichi Medical University using chemical procedures described elsewhere [28]. A stock solution of DHMEQ was prepared

in dimethyl sulfoxide (DMSO) and diluted in culture medium for the appropriate final dose.

Dialysate samples

Dialysate effluents were obtained after the overnight intraperitoneal dwell of the dialysis fluid Dianeal 1.5% (Baxter, McGaw Park, USA) in uremic patients treated with CAPD. Dialysates from 6 patients were collected. Patients had no symptoms of active systemic inflammatory diseases, diabetes mellitus, liver diseases or neoplastic diseases. After drainage the dialysate was spun to remove the floating cells and the supernatant was frozen at -86°C until it was used in the experiments. Before starting the experiment equal volumes of dialysate samples from each patient were mixed to create an "average dialysate" which was used during the experiments on HPMC.

Determination of cell viability

Cells were seeded into 96-well plates at a density of 4×10^3 cells/cm^2 and allowed to attach for 16 h. The cells were growth synchronized by serum deprivation for 4 h and subsequently exposed for 24 h to standard culture medium or standard culture medium supplemented with DHMEQ (final concentration 1 μg/ml, 5 μg/ml, 10 μg/ml). The cells were next incubated in medium containing 1.25 mg/mL of the MTT salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 4 h at 37°C. The formazan product generated was solubilized with lysis buffer (20% sodium dodecyl sulphate and 50% *N*,*N*-dimethylformamide) for 16 h in the dark. After incubation absorbance of the converted dye was recorded at 595 nm with a reference wavelength of 690 nm.

Synthesis of the inflammatory mediators

Synthesis of Interleukin 6 (IL-6), Monocyte Chemoattractant Protein-1 (MCP-1) and Hyaluronan (HA) in mesothelial cells was studied in the cells monolayer, which was exposed to standard culture medium or to culture medium supplemented with DHMEQ in concentrations 1 μg/ml; 5 μg/ml or 10 μg/ml. After 24 hours' incubation supernatant was collected from the wells and stored in aliquots at -80°C until assayed. Concentrations of IL-6, MCP-1 and HA in cell culture supernatants were determined with DuoSet® Immunoassay Development kits (R&D Systems). The release of indicated proteins from the cells was expressed per number of cells.

Measurement of cell proliferation

Mesothelial cells proliferation was examined based on the incorporation of [^{3}H]-thymidine into the DNA of dividing cells. Briefly, mesothelial cells were plated onto 48-well culture dishes at a density of 5×10^4 cells per well and allowed to attach for 24 h. Then, the cells were growth synchronized by serum deprivation for 4 h and subsequently exposed for 24 h to the following media: Serum free medium; Serum free medium mixed 1:1 (v/v) with "average dialysate"; Serum free medium mixed 1:1 (v/v) with "average dialysate" and supplemented with DHMEQ (10 μg/ml).

Additionally tritium labelled-thymidine (methyl-[^{3}H]-thymidine; 1 μCi/mL was added to each well to get its final concentration of 1 μCi/mL. After the 24 hours' exposure to the studied solutions, the cells were harvested with a trypsin-EDTA (0.05%-0.02%) solution and precipitated with 20% (*w/v*) trichloroacetic acid (TCA). The precipitate was washed twice with TCA and dissolved in 0.1 N NaOH. The radioactivity of the cells' lysate was measured in a β liquid scintillation counter (Wallac, Perkin Elmer, Warsaw, Poland). The incorporation of the radiolabelled ^{3}H-methyl-thymidine into DNA of the growing cells was used as an index of their proliferation.

Measurement of collagen synthesis

Mesothelial cells were seeded into 48-well plates, and cultured until the monolayer was present. Then, cells were washed with serum-free medium and 48 hours incubation was started in the following solutions: Serum free medium; Serum free medium mixed 1:1 (v/v) with "average dialysate"; Serum free medium mixed 1:1 (v/v) with "average dialysate" supplemented with DHMEQ (10 μg/ml).

In all groups media were additionally supplemented with β-aminopropionitrile (50 μg/mL), L-ascorbic acid (50 μg/mL) and ^{3}H-Proline (4 μCi/mL). After 48 h incubation supernatant was collected from the wells and the cells were lysed by a repeated freezing and thawing procedure. The collected supernatants and cell

Table 1. Characteristic of primers used in the study

Gene	Name	Gene ID	Amplicon length (bp)	Primer sequence (5'→3')
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	2597	231	F: TTCGTCATGGGTGTGAACC
				R: GATGATGTTCTGGAGAGCCC
HAS1	Hyaluronan synthase 1	3036	393	F: ACTCGGACACAAGGTTGGAC
				R: ACGAGGGCGTCTCTGAGTAG
HAS2	Hyaluronan synthase 2	3037	356	F: TTGGCATCACACCTCATCAT
				R: ACGTGTTGCGAGCTTTCTTT
HAS3	Hyaluronan synthase 3	3038	420	F: ACTGGTACCATCAGAAGTTC
				R: GGGACATGAAGATCATCTCT
IL-6	Interleukin 6	3569	264	F: ATGAACTCCTTCTCCACAAGC
				R: GTTTTCTGCCAGTGCCTCTTTG
MCP1	Monocyte chemoattractant protein 1	6347	153	F: GATCTCAGTGCAGAGGCTCG
				R: TGCTTGTCCAGGTGGTCCAT

lysates harvested from each well were divided into two equal portions which were mixed (1:1 v/v) with: Hanks solution supplemented with N-etylmaleimide (2.5 mM/ml); Hanks solution supplemented with N-etylmaleimide (2.5 mM/ml) and collagenase 0.2 mg/ml).

The prepared supernatants and cell lysates samples were incubated for 4 hours at 37°C. Afterwards the protein in each sample was precipitated with 10% trichloroacetic acid (TCA), after spinning and removal of the supernatant the precipitate was washed with 10% TCA and finally lysed overnight at 4°C in 0.1 N NaOH. The radioactivity of the cells lysates was measured in a β liquid scintillation counter (Wallac, Perkin Elmer, Warsaw, Poland).

Radioactivity of the A samples, without collagenase treatment, reflected total protein synthesis and the difference of radioactivity between samples A and B reflected collagen synthesis.

Gene expression analysis

Mesothelial cells were seeded in 25 cm^2 culture flasks (Nunc A/S, Denmark) and grown until monolayers were established. Afterwards cells from the same donor, in individual flasks, were incubated for 24 h in the following media: Standard culture medium; Standard culture medium + DHMEQ 10 μg/ml; Standard culture medium + Interleukin-1 100 pg/ml; Standard culture medium + Interleukin-1 100 pg/ml + DHMEQ 10 μg/ml.

After exposure, total RNA from cells was isolated using the TRIzol reagent (Invitrogen) method according to the manufacturer's instruction. RNA samples were treated with DNase I using DNA-free DNase Treatment and Removal Reagent (Ambion). RNA quality and concentration were assessed by spectrophotometry using a NanoDrop (NanoDrop, Thermo Scientific, DE, USA).

One microgram of total RNA was reverse-transcribed to cDNA using the BioScript All-in One cDNA Synthesis Super Mix (Biotool.com). Relative levels of mRNA were examined using Sybr green real-time quantitative PCR (Applied Biosystems) and normalized to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Specific primers for the amplification of each gene were designed using Primer-BLAST [29]. Characteristic of primers used in the study is presented in **Table 1**. The PCR parameters were as follows: initial denaturing for 10 min at 95°C, followed by 35 cycles of denaturation (95°C for 60 s), annealing (50-60°C, depending on the primers used for 45 s) and extension (72°C for 45 s). After completed real-time PCR reactions, a melting curve analysis was performed for each sample to confirm that a single, specific product was generated. Relative gene expression was calculated using the 2-ΔΔCt method [30]. All PCRs samples were performed in hexaplicate, and the data are presented as means ± SD.

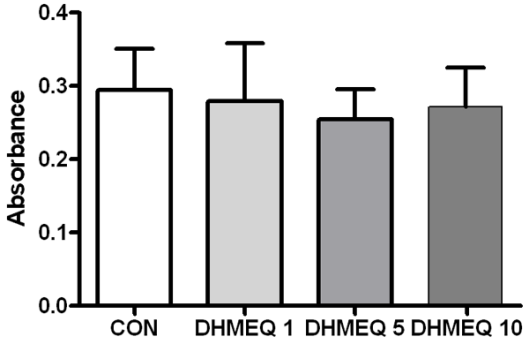

Figure 1. Results of the MTT assay on human peritoneal mesothelial cells exposed during 24 hours to standard culture medium (CON) or culture medium supplemented with DHMEQ in concentrations 1, 5 and 10 μg/ml (n=6).

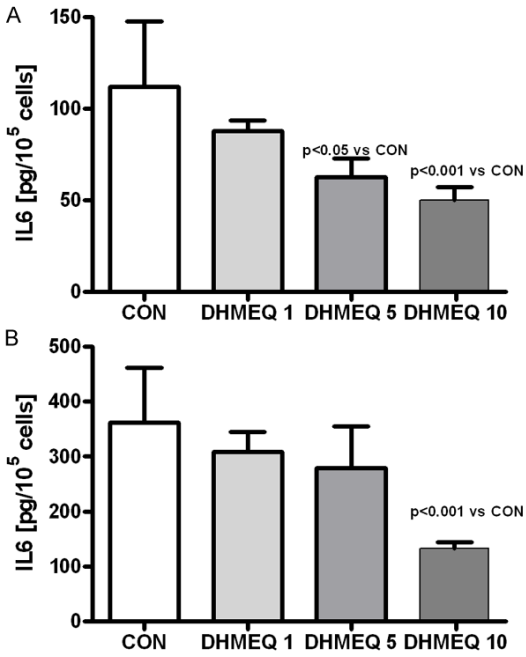

Figure 2. Synthesis of the IL-6 in unstimulated (A) or stimulated with interleukin-1 (100 pg/ml) (B) human peritoneal mesothelial cells exposed during 24 hours to standard culture medium (CON) or culture medium supplemented with DHMEQ in concentrations 1, 5 and 10 μg/ml) (n=6).

Statistical analysis

Results are presented as means ± SD. For comparison of two matched samples (effect of interleukin 1 on synthesis of the inflammatory mediators in mesothelial cells) the Wilcoxon test was used. In other cases statistical analysis was performed with the Friedman test with post hoc analysis performed with Dunns test. A

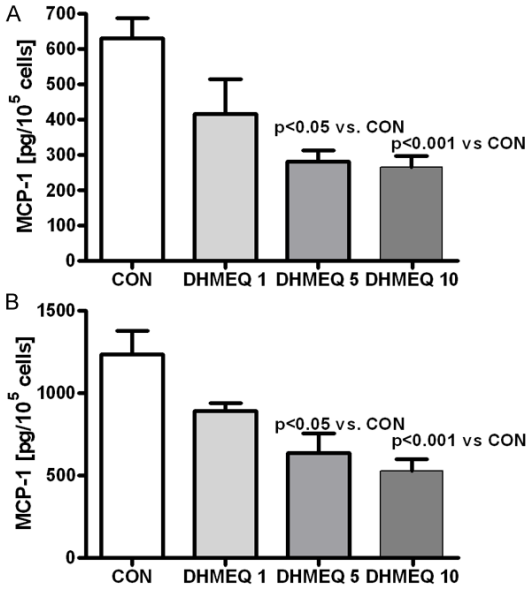

Figure 3. Synthesis of the MCP-1 in unstimulated (A) or stimulated with interleukin-1 (100 pg/ml) (B) human peritoneal mesothelial cells exposed during 24 hours to standard culture medium (CON) or culture medium supplemented with DHMEQ in concentrations 1, 5 and 10 μg/ml) (n=6).

p value less than 0.05 was considered as significant.

Results

The results from our study confirm that NF-κB inhibitor-DHMEQ modifies the function of human peritoneal mesothelial cells in conditions of *in vitro* culture. No signs of toxicity of DHMEQ towards the mesothelial cells were detected with the MTT test, when the cells were exposed to that compound added to culture medium in the range of concentrations from 1 μg/ml to 10 μg/ml (**Figure 1**).

HMPC cultured *in vitro* produced IL-6, MCP-1 and hyaluronan which were released from the cells and then detected in medium. Stimulation of HPMC with interleukin-1 (100 pg/ml) resulted in increased synthesis of IL-6 by 222%, MCP-1 by 96% and hyaluronan by 132%. DHMEQ used as an additive to the medium caused a dose dependent inhibition of IL-6 (**Figure 2**), MCP-1 (**Figure 3**) and hyaluronan (**Figure 4**) synthesis in both unstimulated and stimulated mesothelial cells. DHMEQ used at concentration 10 μg/ml inhibited synthesis of IL-6 by 55% in unstimulated HPMC and by 63% in stimulated cells (**Figure 2**). Respective val-

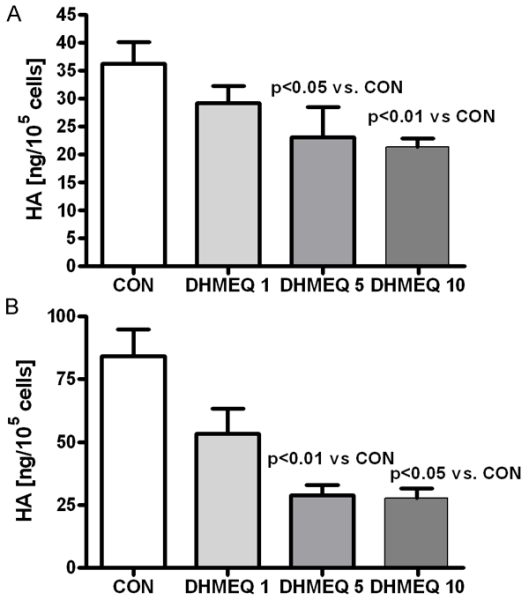

Figure 4. Synthesis of the hyaluronan in unstimulated (A) or stimulated with interleukin-1 (100 pg/ml) (B) human peritoneal mesothelial cells exposed during 24 hours to standard culture medium (CON) or culture medium supplemented with DHMEQ in concentrations 1, 5 and 10 μg/ml) (n=6).

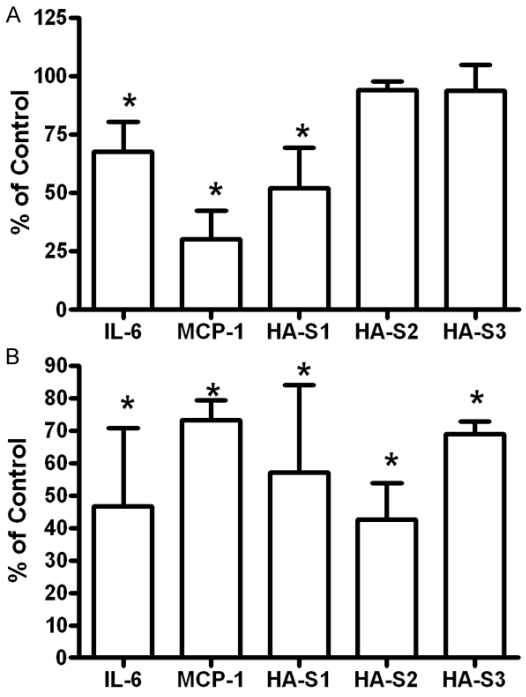

Figure 5. Expression of genes for IL-6, MCP-1, HA-S1, HA-S2 and HA-S3 after exposure of the cells to DHMEQ 10 μg/ml in unstimulated mesothelial cells (A) and stimulated with interleukin-1 (100 pg/ml) mesothelial cells (B). Results are presented as % of control group in which mesothelial cells were not expose to DHMEQ (*P<0.05 vs. Control) (n=6).

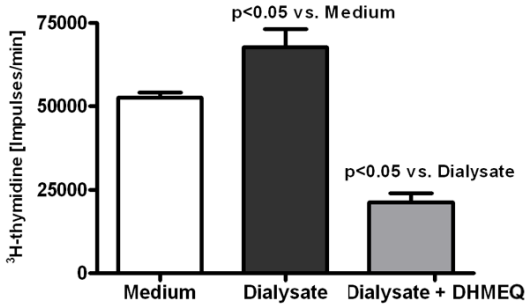

Figure 6. Incorporation of ^{3}H-thymidine into human peritoneal mesothelial cells exposed to medium (Medium) or to medium mixed (1:1 v/v) with effluent dialysate ± DHMEQ 10 μg/ml (n=8).

ues for MCP-1 were inhibition by 58% and by 57% (**Figure 3**) and for hyaluronan inhibition by 41% and by 67% (**Figure 4**).

Exposure of the mesothelial cells for 24 hours to DHMEQ resulted in decreased expression of genes for IL-6 and MCP1 both in unstimulated and stimulated cells (**Figure 5**). Expression of HA-S1 genes was suppressed by DHMEQ both in unstimulated and stimulated cells but expression of HA-S2 and HA-S3 only in stimulated cells (**Figure 5**).

The mixture of effluent dialysates obtained from CAPD patients accelerated proliferation of HPMC by 28% but that stimulatory effect was reduced by 69% when DHMEQ at concentration 10 μg/ml was simultaneously added to culture medium (**Figure 6**). Dialysate stimulated the synthesis of total proteins in HPMC by 30% and that effect was not modified by DHMEQ at concentration 10 μg/ml (**Figure 7**). We also observed a trend for higher synthesis of collagen in HPMC exposed to the dialysates but the amount of produced collagen was reduced by 40% when DHMEQ 10 μg/ml was simultaneously present in culture medium (**Figure 7**).

Discussion

Peritoneal dialysis induces intraperitoneal inflammation, which in the long term may lead to fibrosis of the peritoneum and loss of the functional properties of that biological dialysis membrane. Activation of the mesothelial cells and their epithelial-to-mesenchymal transition are important steps resulting in the progression of peritoneal structural damage and membrane failure [31]. Therefore it can be suggested that peritoneal mesothelial cells should be a

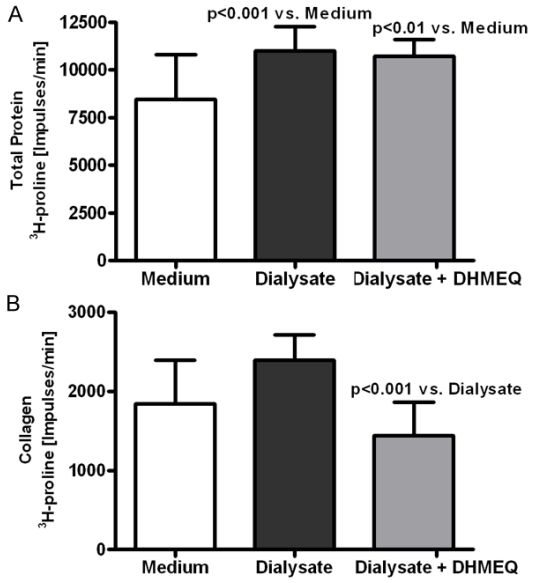

Figure 7. Synthesis of total protein (A) and collagen (B) in human peritoneal mesothelial cells cultured in medium (Medium) or in medium mixed (1:1 v/v) with the effluent dialysate (Dialysate) ± DHMEQ 10 μg/ml (n=8).

target of the therapeutic approaches aimed at slowing the deterioration of the peritoneal function during chronic dialysis.

Inhibition of NF-κB activity in mesothelial cells results in the suppression of their inflammatory profile [32] and epithelial to mesenchymal transition [33]. The results of our experiments show that DHMEQ, a new NF-κB inhibitor decreases the inflammatory reaction in human peritoneal mesothelial cells as reflected by suppressed activity of genes regulating synthesis of IL-6 and MCP-1 (**Figure 5**) and in consequence reduced the release of IL-6 and MCP-1 from unstimulated and stimulated cells (**Figures 2** and **3**). Various components of the dialysis fluid such as osmotic factors (ie. glucose) or glucose degradation products stimulate NF-κB in the mesothelium which results in increased release of the inflammatory mediators and may propagate an intraperitoneal inflammatory reaction [14, 17]. The inhibition of these effects by DHMEQ seems to be beneficial, because it can stop both the intraperitoneal influx of leukocytes and inflammation [4].

DHMEQ also inhibited hyaluronan synthesis and its release from the mesothelial cells, especially from cells stimulated with IL-1 (**Figure 5**). Hyaluronan plays an important role in the peritoneum, regulating its hydration and permeability, and protecting the mesothelial cells [34]. In uraemic patients treated with peritoneal dialysis increased submesothelial expression of hyaluronan is increased which may be caused by chronic intraperitoneal inflammation [35]. The consequences of this effect are not well known, because there is a lack of research in this area. Extrapolating data from other experimental models it can be said that it may promote fibrosis [36] and in such cases the observed effect of DHMEQ in our experimental cells may be beneficial. On the other hand, the supplementation of the dialysis fluid with hyaluronan in rats maintained on chronic peritoneal dialysis helped to maintain the structure and function of the peritoneum as the dialysis membrane [37]. In another experiments we found that the synthesis of collagen in mesothelial cells exposed in vitro to effluent dialysates from CAPD patients was inversely proportional to hyaluronan concentrations in the studied samples (unpublished data). Further studied are required to evaluate the positive and negative effects caused by the DHMEQ-induced inhibition of hyaluronan synthesis in peritoneal mesothelial cells.

Effluent dialysate obtained from uraemic patients treated with peritoneal dialysis stimulated the proliferation of mesothelial cells in *in vitro* culture. We found previously that the intensity of the stimulatory effect of the effluent dialysates on the proliferation of mesothelial cells correlated with their concentration of interleukin-6 (unpublished data). In other experimental models it was found that IL-6 stimulates the growth of endothelial cells and promotes the epithelial to mesenchymal transition of cholangiocarcinoma cells [38, 39]. The inhibition of such processes in peritoneal mesothelial cells by DHMEQ, as seen in our study, may help to preserve the structure of the peritoneum in conditions of chronic peritoneal dialysis.

Fibrosis of the peritoneum is a process which appears, with varying intensity, in all patients treated with chronic peritoneal dialysis [4]. In our experiments effluent dialysate stimulated the synthesis of both total protein and collagen in mesothelial cells (**Figure 7**). We found that DHMEQ did not change the stimulatory action of the dialysate on total protein synthesis but

reduced the synthesis of collagen. Similar effects caused by NF-κB inhibition with pioglitazone in rat mesothelial cells were described by Zhou et al [19]. Our observations confirm a previous report about the inhibitory effect of DHMEQ on collagen synthesis in keloid fibroblasts [40]. The inhibition of collagen synthesis within the peritoneum could result in the slower process of the peritoneal fibrosis in CAPD patients.

In summary, we found that DHMEQ is harmless to mesothelial cells as evaluated with the standard cytotoxic MTT test and inspection of cells morphology. DHMEQ suppresses inflammatory reaction in human peritoneal mesothelial cells and modulates the effect of the effluent dialysates from CAPD patients on growth and collagen synthesis in these cells. The majority of the observed effects may be beneficial in conditions of peritoneal dialysis. However further studies are required to find out what the long term local and systemic effect of treatment with DHMEQ could be.

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Disclosure of conflict of interest

None.

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