Hydralazine inhibits human peritoneal mesothelial cell proliferation and collagen synthesis

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Abstract The integrity of the mesothelial layer is essential for both defence and solute transport in continuous ambulatory peritoneal dialysis (CAPD). The human peritoneal mesothelial cell (HPMC) culture has been shown to be a very useful tool to study the peritoneal mesothelial stem cell behaviour. We investigated whether hydralazine, an antihypertensive agent frequently used, might affect HPMC growth and collagen synthesis. HPMCs were cultured from specimens of human omentum by enzymatic disaggregation of omentum. HPMC growth was evaluated by modified methyltetrazolium (MTT) assay. Cell viability was confirmed by trypan blue exclusion and lactate dehydrogenase assay. Collagen synthesis was measured by 3H-proline incorporation into pepsin-resistant, salt-precipitated collagen. Intracellular cAMP levels were measured by enzyme immunoassay. The procollagen α1 (I) mRNA expression was evaluated by Northern blot analysis. Hydralazine inhibited serum-stimulated HPMC growth in a dose-dependent manner. The maximal inhibition was 93% at a concentration of 100 μg/ml. Hydralazine inhibited collagen synthesis in confluent mesothelial cells (47% inhibition at a concentration of 100 μg/ml). The procollagen α1 (I) mRNA expression was also decreased by hydralazine (about 50% decrease at 100 μg/ml). These effects may be due to the phosphodiesterase inhibition property of hydralazine to increase intracellular cAMP levels. These data suggest that the use of hydralazine in CAPD patients may affect peritoneal membrane function and integrity.

Key words: cAMP; collagen; hydralazine; peritoneal dialysis; peritoneal mesothelial cell

Introduction

Continuous ambulatory peritoneal dialysis (CAPD) has been used more frequently in recent years as treatment for end-stage renal disease. The integrity of the mesothelial layer is essential for both defence and solute transport. The peritoneal mesothelial cell layer is under continuous sloughing and regeneration processes during CAPD. The new mesothelium that develops after injury arises from peritoneal mesothelial stem cells [1–3]. Because mesothelial cells in culture have been shown to possess the same immunocytochemical markers as stem cells (co-expression of cytokeratins and vimentin) [4,5], this culture system can be used as a tool to study the behaviour of peritoneal mesothelial stem cells. Any agent which interferes with mesothelium stem cell proliferation may be harmful to the peritoneal membrane. Therefore, any medication in patients with CAPD should in principle be evaluated with respect to the effect of the drugs on peritoneal mesothelial stem cells.

Hydralazine is a frequently used drug in patients with end-stage renal disease, to control hypertension. It is still unknown whether the use of hydralazine in patients having CAPD affects peritoneal membrane function. Therefore, we investigated the effects of hydralazine on the behaviour of human peritoneal mesothelial cells (HPMCs) in culture.

Subjects and methods

Materials

Trypsin-EDTA, RPMI-1640 medium, glutamine and trypan blue were obtained from Gibco (Grand Island, New York, USA). Culture flasks and plates were purchased from Corning (Corning, New York, USA) and precoated with a density of 1.6 μg/cm² Vitrogen 100® (Celtrix Lab., Palo Alto, CA, USA) before loading cells. Fetal calf serum (FCS) was obtained from Biochrom KG (Berlin, Germany). Triton X-100, bovine serum albumin, N⁰-monomethyl-L-arginine (L-NMMA), N⁰, 2'-O-dibutyryladenosine 3', 5'-cyclic monophosphate (DBcAMP), 3-isobutyl-1-methylxanthine (IBMX), prostaglandin E₂ (PGE₂), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), hydralazine, penicillin, streptomycin, insulin, ascorbic acid, β-aminopropionitrile, and pepsin-containing acetic acid were bought from Sigma (St Louis, Missouri, USA). 3H-proline was obtained from Nen Dupont (Boston, Massachusetts, USA).
The enzyme immunoassay (EIA) kits for cyclic adenosine monophosphate (cAMP), 6-keto-prostaglandin F 1α, (6-keto-PGF 1α) and prostaglandin E 2 (PGE 2) were brought from Cayman Chemical Co. (Ann Arbor, MI, USA). Monoclonal antibodies to human desmin, vimentin, cytokeratin, and factor VIII-related antigen were from Dako Co. (Kyoto, Japan). Agents used for Northern blot analysis were from Boehringer Mannheim GmbH (Mannheim, Germany) unless otherwise specified.

**HPMC culture**

Specimens of human omentum were obtained from abdominal surgical procedures with informed consent. These patients were operated for elective abdominal surgery and the omentum was normal. The method of enzymatic disaggregation of omentum was used as previously described [6]. Briefly, a piece of omentum was washed three times in sterile phosphate-buffered saline (PBS) and then incubated with 15 ml of trypsin-EDTA (0.125%) for 20 min at 37°C with continuous rotation. After incubation the omentum and the suspension were centrifuged at 50 g for 5 min at 4°C. The supernatant was discarded together with the omentum and the cell pellet was washed in RPMI-1640 medium containing 20% FCS, penicillin 100 U/ml, streptomycin 100 μg/ml, and insulin 30 μg/ml. After another centrifugation in the same condition, the cells were resuspended in the same medium and seeded into a 75 cm² flask. After 2-4 days, the cells became confluent and were subcultured with medium containing 10% FCS. The cells were initially bipolar or multipolar but became cobblestone-like in appearance upon confluence. Mesothelial cells were identified by the presence of vimentin and cytokeratin and the absence of desmin and factor VIII-related antigen using the immunofluorescence method. All experiments listed below were performed in passage 1-3 cells and repeated at least three times using cells from different subjects.

**Cell proliferation assay**

The modified MTT assay was used [7]. The MTT assay is based on the ability of living cells to take up and reduce MTT to form the blue formazan product by their mitochondria. The amount of MTT taken up by HPMCs (measured by absorbance at 570 nm) was found to vary linearly with the cell number, ranging from 4000 cells/well to 1.28 x 10⁵ cells/well in a 96-well plate and grown to a confluent monolayer. The medium was then replaced by 0.5 ml RPMI-1640 medium after washing twice in warm PBS. Various concentration of hydralazine were added, and 0.015% Triton X-100 was added as a positive control. The plates were incubated for 1.3 and 5 days and then the LDH activity of the supernatant was measured by the enzymatic method [12]. All experiments were repeated four times.

**Collagen synthesis**

HPMCs were plated down at a density of 2.5 x 10⁴ cells/well in a 96-well plate in 200 μl medium supplemented with 10% FCS. After incubation for 48 h, the cells became confluent. The medium was then replaced by 10% FCS-supplemented medium containing 50 μg/ml ascorbic acid with or without different concentrations of hydralazine. After an additional incubation of 48 h, cultures were labelled with 0.5 μCi ³H-proline (100 Ci/mmole, ICN) and 50 μg/ml β-aminopropionitrile for the final 24 h of incubation [13]. ³H-proline incorporation into pepsin-resistant, salt-precipitated collagen was determined as previously described [14]. After termination of incubation, ³H-proline-labelled collagen was extracted from each well by the addition of pepsin-containing acetic acid (final concentration 1 mg/ml in 1 M acetic acid) and purified by successive salt precipitation at acid and neutral pH in the presence of carrier collagen. The final precipitates were dissolved in 0.5 M acetic acid, incorporated into a scintillation cocktail and counted using a liquid scintillation counter. Cell numbers were simultaneously counted using the MTT assay in identically treated microwells and the collagen synthetic amount was factored by the same treated MTT. All experiments were done in triplicate.

**Northern blot analysis**

HPMCs were cultured in a 50 cm² dish with 10% FCS-containing RPMI to a confluent monolayer. The supernatants were then replaced with 10% FCS-containing RPMI with various concentrations of agents. At different time intervals, the total RNA was isolated using the acid guanidinium thiocyanate-phenol–chloroform method, as described by Chomczynski and Sacchi [15]. The amount of RNA was quantified using spectrophotometry with absorbance at 260 nm. Ten micrograms of RNA were then electrophoresed on a 1% agarose gel containing 1.0 M formaldehyde in MOPS buffer (0.2 M morpholinopropanesulfonic acid, 0.05 M Na acetate, 0.01 M EDTA). Equivalency of sample loading and lack of degradation were verified by ethidium

**Cell viability test**

To exclude a toxic effect of hydralazine on HPMCs, cell viability was assessed by the trypan blue exclusion method in 24-well plates, after incubating at various doses of hydralazine for 2-5 days [10]. The number of dead cells from the supernatant in each well was counted and the proportion of dead cells of the cells adhering to the plates was also recorded by adding trypan blue directly to the well. All samples were done in triplicate.
bromide staining of 28S and 18S rRNA bands. The RNA was transferred to nylon membrane by overnight capillary action and followed by fixation in UV cross-linker.

Hybridization of the Northern blots was performed with a cRNA probe for 1.5-kb human type I collagen [procollagen α1 (I)]. The cRNA probes were transcribed using phage T3 and T7 DNA-dependent RNA polymerase in the presence of digoxigenin-UTP as described by the supplier, from subcloned templates corresponding to a 1.5-kb EcoRI fragment from human procollagen α1 (I) cDNA (HF677, ATCC, Rockville, MD, USA). The RNA blots were prehybridized for 3 h at 65°C in a hybridization solution composed of 50% formamide, 5 x SSC (1 x = 120 mM NaCl, 15 mM Na citrate, 13 mM KH2PO4 and 1 mM EDTA, pH 7.2), 0.1% Na lauryl sarcosine, 2% Na dodecyl sulphate (SDS) and 5% blocking reagent. After overnight hybridization at 65°C with 20 ng/ml probe in the same solution, the blots were washed in 2 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS at 65°C. Finally, the blots were developed using alkaline phosphatase-conjugated anti-digoxigenin antibody at Lumiphos 530 according to the manufacturer’s directions. The signal intensity recorded on X-ray film was quantified by computerized densitometry. The blots were also probed with a cRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control for variation in RNA loading and transfer.

Intracellular cAMP measurement

Three hundred thousand HPMCs per well were loaded into a 6-well plate and cultured by medium supplemented with 10% FCS. After cells became confluent, they were washed twice with warm RPMI-1640 medium and then mixed with various concentrations of agents. After 5 min of incubation, the supernatants were discarded and ice-cold 95% methanol/5% formic acid solution was added to each well. After 30 min of incubation at 4°C, the supernatants evaporated and cAMP was measured using EIA kits. The cells in wells were lysed with 0.1 N NaOH and protein content was measured with the bicinchoninic acid assay, using bovine serum albumin as standard [16]. All experiments were done four times.

PGE2 and 6-keto-PGF1α assay

Seventy thousand HPMCs per well were loaded into a 24-well plate and cultured by medium supplemented with 10% FCS. After cells became confluent, they were washed twice with warm RPMI-1640 medium and then various concentrations of agents were added the plates incubated for 1 h. The supernatants were collected and PGE2 and 6-keto-PGF1α levels were assayed using EIA kits. The cell number of each well was determined by MTT assay. All experiments were done four times.

Nitrite assay

Nitric oxide (NO) production was determined by measurement of nitrite (as an end product of NO metabolism) in the culture supernatant [17]. Three hundred thousand HPMCs per well were loaded into a 6-well plate and cultured by medium supplemented with 10% FCS. After cells became confluent, the medium was replaced by phenol red-free RPMI-1640 containing 2.5% FCS with various concentrations of agents. After a 24-h incubation, the supernatants were collected to measure nitrite amount by absorbance at 540 nm in a spectrophotometer after reacting with Greiss reagent [18]. The cell number of each well was determined by MTT assay. All experiments were done four times.

Statistical analysis

All data are expressed by mean±SD. The comparison of dose effect was done by a one-way ANOVA followed by Student’s t-test. A P value less than 0.05 was considered as significant.

Results

At concentrations below 100 μg/ml (0.51 mM), hydralazine caused a dose-dependent inhibition of HPMC growth (Fig. 1). The percentage inhibition for concentrations from 3 to 100 μg/ml was 9% to 93%. Cell viability was confirmed by the trypan blue test, which

![Fig. 1. Hydralazine inhibits HPMC growth. (A) Time response curve. Various concentrations of hydralazine [0 (■), 12 (▲), 25 (●), and 100 (○) μg/ml] were added after overnight plating of HPMCs and the cells were further incubated for 1–5 days. (B) Dose response curve. Cells were incubated for 5 days after addition of various concentrations of hydralazine. All data are expressed as the means (SD) of three experiments conducted × 3. *P<0.05 vs control.](image-url)
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Fig. 2. Collagen synthesis of HPMCs in the presence of various concentrations of hydralazine. Results are expressed as percentage of the control. All data are expressed as means (SD) of three experiments conducted in triplicate. *P<0.05 vs control.

showed there were no differences in supernatant and adherent cells fractions between control and hydralazine-treated wells. The dead cells of each well made up less than 1% of the total cells. The levels of LDH were not elevated after 1-, 3-, and 5-day incubation periods in hydralazine-treated wells, which expelled the cell membrane damage by hydralazine on the HPMCs (data not shown).

Collagen synthesis of HPMCs was suppressed by hydralazine in a dose-dependent manner (Fig. 2; control: 41.63 ± 2.45 dpm/10³ cells; 25 µg/ml hydralazine: 43.80 ± 1.38 dpm/10³ cells; 50 µg/ml hydralazine: 34.07 ± 6.77 dpm/10³ cells; 100 µg/ml hydralazine: 19.68 ± 1.67 dpm/10³ cells; P < 0.05 among control and the two higher doses). The mRNA expression of type I collagen was measured after adding hydralazine. Northern blot analysis demonstrated two bands at 4.8 and 5.8 kb. The results of densitometry showed that hydralazine decreased procollagen α1 (I) mRNA expression while GAPDH mRNA was not affected. The time course and dose–response effect of hydralazine on procollagen α1 (I) mRNA are shown in Fig. 3. Maximal decrease of procollagen α1 (I) mRNA levels was observed at 3 h after addition of 100 µg/ml hydralazine (about 50% decrease).

Table 1. The changes of intracellular cAMP (pmol/mg protein) in HPMCs

<table>
<thead>
<tr>
<th>Without IBMX 0.5 mM</th>
<th>With IBMX 0.5 mM</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
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<tr>
<td>Hydralazine 25 µg/ml</td>
<td>5.1 ± 1.0</td>
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<tr>
<td>Hydralazine 50 µg/ml</td>
<td>8.2 ± 1.3*</td>
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<tr>
<td>Hydralazine 100 µg/ml</td>
<td>21.4 ± 6.3*</td>
</tr>
<tr>
<td>PGE₁ 10 µM</td>
<td>37.3 ± 16.8*</td>
</tr>
<tr>
<td>Hydralazine 100 µg/ml + PGE₁ 10 µM</td>
<td>233 ± 29**</td>
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<td></td>
<td>616 ± 38**</td>
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cAMP levels are expressed as means ± SD of three experiments conducted x4.
* P<0.05 vs control.
† P<0.05 between any two measurements.

The intracellular cAMP levels are shown in Table 1. cAMP levels were elevated in a dose-dependent manner by hydralazine. PGE₁ increased cAMP level at 10 µM, which was potentiated by 100 µg/ml hydralazine or 0.5 mM IBMX. When various concentrations of hydralazine were combined with 0.5 mM IBMX, there was a slight elevation of cAMP only at the highest concentration of hydralazine. DBcAMP, a lipophilic cAMP...
Fig. 4. MTT assay on the 5th day after adding DBcAMP. DBcAMP inhibited HPMC proliferation in a dose-dependent manner. All data are expressed as means (SD) of three experiments conducted x 5. *P < 0.05 vs control.

Collagen I/GAPDH Ratio 1.72 1.69 0.93 1.44 2.39 1.23 1.29 1.51 2.08

Fig. 5. Northern blot analysis of mRNA isolated form HPMCs incubated with or without DBcAMP. The RNA blots were hybridized with procollagen α I (I) and GAPDH cRNA probes. Cells were treated with DBcAMP 3 mM for 24 h (1), 16 h (2), 7 h (3), and 3 h (4). Cells were treated with DBcAMP 1 mM for 3 h (5), 7 h (6), 16 h (7), and 24 h (8). C: control.

Discussion

The most frequent complication during CAPD is peritonitis. During the acute phase of peritonitis, necrosis and sloughing of the mesothelial cell layer occur, leaving a denuded surface. Regeneration of the mesothelial layer depends on active proliferation and upward migration of underlying mesothelial stem cells [19]. If for any reason mesothelial recovery of the peritoneal surface was delayed, the underlying stem cells would be continuously exposed to growth factor stimulation which might lead to peritoneal fibrosis [19]. Proliferation of peritoneal mesothelial stem cells accompanied by fibrous tissue overproduction is currently thought to be the most likely pathophysiologic process of peritoneal fibrosis [20,21]. Studies of the composition of peritoneal fibrotic lesions suggest that these lesions represent an accumulation of type I and III collagen as well as other matrix proteins such as fibronectin [22]. Agents which can inhibit peritoneal mesothelial stem cell proliferation and/or collagen synthesis may be beneficial to the prevention or retarding of the progression of peritoneal fibrosis. In this study, we demonstrated hydralazine-inhibited HPMC growth and collagen synthesis in vitro. It is reasonable to use hydralazine in those conditions associated with mesothelial cell overgrowth, such as sclerosing peritonitis.

The pharmacodynamic mechanism of hydralazine has been studied extensively, but is still controversial. Increase of cAMP through inhibiting phosphodiesterase is one theory [23,24]. Other mechanisms such as increased cGMP through NO release [25,26] and stimulating prostaglandins synthesis [27] have been proposed. However, some studies failed to show any changes of cAMP or cGMP levels after hydralazine treatment [28–31]. In our study, we did not detect any increase of PGE2 or PG12 (the two major products of arachidonic acid metabolism in HPMCs [6]) synthesis. The antiproliferative effect of hydralazine was not altered by L-NMMA, nor was nitrite elevated after hydralazine treatment. These data imply that the anti-mitogenic effect of hydralazine is not mediated by the arachidonic acid pathway or by increased NO synthesis. We demonstrated an increase of cAMP after hydralazine treatment, and hydralazine could potentiate the effect of PGE2 on cAMP elevation, as IBMX did. When hydralazine and IBMX were combined the cAMP elevation was only slightly increased over IBMX alone. These results indicate that the mechanism of hydralazine was similar to IBMX as a phosphodiesterase inhibitor. We also showed that DBcAMP could inhibit HPMC growth and decrease procollagen α I (I) mRNA synthesis, so the hydralazine effect may be through the cAMP pathway.

The usual plasma level of hydralazine after a 100-mg oral dose is about 1.0 μg/ml [32]. Our study used a higher dose of hydralazine in the culture system to show its effects on HPMCs. However, the plasma level might not reflect the tissue level, so many in vitro studies on hydralazine [23,24,28–31] use the same dose range (10 μM to 1 mM) as our study.

In conclusion, hydralazine inhibits HPMC growth and collagen synthesis in vitro through the cAMP pathway, which may affect its use in the CAPD patient.
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However, the in vivo effects of hydralazine on peritoneal membrane need further study.

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