

Spontaneous VEGF Production by Cultured Peritoneal Mesothelial Cells from Patients on Peritoneal Dialysis

Long-term peritoneal dialysis (PD) may result in increased peritoneal vascularization secondary to neovascularization (1). Vascular endothelial growth factor (VEGF) is the main factor known to participate in new vessel formation (2). Peritoneal VEGF production has to be an etiologic factor in peritoneal neovascularization due to the relatively higher levels found in peritoneal effluent than in plasma (3). The exact origin of peritoneal effluent VEGF is not known. Recently, pleural mesothelial cells (MC) have been shown to produce small amounts of VEGF (4). Also, VEGF has been detected in peritoneal capillary endothelium and surrounding tissue in long-term PD patients (5). Finally, the glucose degradation product (GDP) carboxymethyl lysine has been demonstrated to be co-localized with VEGF in the mesothelial layer in chronic PD patients (6). These authors have also shown the production of VEGF by rat MC exposed to GDP.

Many usual conditions intrinsic to PD are related to the VEGF present in the peritoneum in these patients. Our hypothesis is that human MC might be a source for VEGF. To study their potential VEGF production, we determined VEGF levels in conditioned medium during ex vivo culture of MC isolated from the peritoneal effluent of active PD patients.

PATIENTS AND METHODS

Patients and Peritoneal Characteristics: We assessed peritoneal antecedents and peritoneal function by determining the mean number of prior peritonitis episodes and the peritoneal transport parameters [mass transfer coefficients (MTC) of urea and creatinine and net ultrafiltration during a 4-hour kinetic study with 2.27% glucose] (7) in 21 patients. In addition, the accumulated peritoneal dose of glucose administered during the complete PD period was calculated for each patient.

Mesothelial cells (8) were isolated from nocturnal peritoneal effluent incubated in hanging PD bags for 3 - 4 hours at 37°C. The residual effluent was removed by vacuum suction with a sterile pipette, leaving approximately 200 mL in the bottom. Cells were resuspended in this volume, transferred to four 50-mL tubes, and washed twice with phosphate-buffered saline by centrifugation at 1500 rpm for 20 minutes. Then they were seeded in 25-cm² tissue culture flasks and incubated at 37°C in a humidified atmosphere with 5% CO₂, and maintained in M-199 (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 20% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2% Biogro-2 (containing insulin, transferrin, ethanol amine, and putrescine) (Biological Industries). After reaching confluence, cells were detached with trypsin/EDTA,

resuspended, and seeded in 24-well plates at 2×10^4 cells/well. Incubation was performed as before; the medium was renewed every 3 - 4 days.

None of the cultures used to evaluate VEGF production showed bacterial or fungal contamination.

Mesothelial cells were identified at isolation from effluent and after trypsinization. Morphology was assessed in cytospin optical microscopy slides (500 rpm) stained with May-Grünwald-Giemsa. Mesothelial cells were thus easily distinguished from macrophages, lymphocytes, and neutrophils by their morphological features defined under light microscope. No atypical or giant cells were observed in these cultures. Mesothelial cells were characterized by immunohistochemical staining, identifying MC by their positive staining for cytokeratin and negative staining for CD45 (hematopoietic cells), HLA-DR (macrophages and lymphocytes), and vWF (endothelial cells). All antibodies were from Dako (Glostrup, Denmark).

VEGF₁₆₅ Determinations: VEGF was measured in the supernatant of 21 cultures of MC. VEGF determination in supernatant was performed at day 13 of seeding in 24-well plates, since the proliferation plateau usually starts at this time while MC simultaneously form a monolayer reaching confluence. This time avoids vigorous cell proliferation, which is a consequence of cell subconfluence state. After 48 hours of serum deprivation, supernatant was taken for analysis. VEGF₁₆₅ was determined by enzyme immunoassay (ELISA) with a kit from R&D Systems, Inc. (Minneapolis, MN, U.S.A.).

Statistical Analysis: Data are expressed as mean \pm SD. The Mann-Whitney test was used for data comparisons as a nonnormal distribution of the results was found. Regression analysis was performed with the Spearman test.

RESULTS

VEGF was detected in the supernatant from all 21 independent cultures of MC taken from PD bags and grown in culture. All the values observed were within the detection range for the ELISA kit, with mean VEGF levels of 548 ± 518 pg/mL (range 59 - 1747 pg/mL). When peritoneal data and cell counts from quartile 25 and quartile 75 were compared, no significant differences were found (Table 1). Although VEGF is also a vascular permeability factor, the levels found in conditioned medium from MC in culture are not related to peritoneal permeability to small molecules.

Due to the wide range of values, the entire group was artificially divided for supernatant VEGF levels higher ($n = 12$) and lower ($n = 9$) than 200 pg/mL. There was a trend toward a longer stay on PD in patients with lower supernatant levels. The mesothelial cell count on day 13 was similar for the two groups ($65\ 699 \pm 28\ 738$ vs $74\ 930 \pm 39\ 245$, $p = 0.88$); the accumulated peritoneal glucose load was also similar (72.06 ± 70.73 kg vs 122.41 ± 211.78 kg, $p = 0.86$).

We found no overall correlation between supernatant VEGF levels and age, sex, time on PD, solute transport characteristics (Table 2), ultrafiltration rate, or accumulated dose of glucose. The MC count at day 13 was not significantly correlated with VEGF levels in the supernatant ($r = -0.33$, $p = 0.13$, NS). Furthermore, correction of supernatant VEGF levels according to the cell density at day 13 showed no significant correlation with any of the independent variables. We found no differences in supernatant VEGF levels between the different types of MC morphologies.

DISCUSSION

In the present study, we report the capacity of peritoneal MC, cultured in vitro, to produce VEGF, suggesting that MC may contribute to the neoangiogenesis observed in some PD patients. This study represents a direct demonstration of VEGF production by human peritoneal MC in culture, and provides an explanation for the significant amounts of VEGF found in peritoneal effluent (3) and for its presence at the mesothelial level (6). However, these findings do not demonstrate the constitutive production of VEGF by MC from nondialyzed peritoneum. VEGF production by MC might be the result of an interaction with the continuously administered dialysate. In fact, pleural MC in culture from patients with cardiac insufficiency produce VEGF in amounts below the range of technique detection (4), much lower than the levels we have found in peritoneal MC. The extreme differences between the VEGF levels produced by each cell population could be determined by the particular situation of peritoneal MC in PD patients. The amounts produced by peritoneal MC are closer to the amounts produced by malignant pleural MC than to those produced by nonmalignant cells (4). No information has been found on VEGF production by MC lines.

Although other peritoneal cells, such as macrophages, are able to produce VEGF (9), there is no possibility of interference by these cells in our study. By our methodology (8), macrophages initially collected from the peritoneal effluent are progressively eliminated by washing, as they do not adhere to the flask surface.800

We have demonstrated by analysis of supernatant from cultured MC that peritoneal MC can produce VEGF. This production is entirely spontaneous, although we do not know whether it might be a response to other stimuli. VEGF supernatant levels were independent of the growth rate and number of MC. There was a tendency toward an inverse relationship between this number and the VEGF level, but it was not statistically significant. The great variation in levels found in our series is remarkable and is not explained by any of the variables examined. The analysis by extreme groups of values showed no significant differences. The morphology of MC observed

after their growth in T-25 flasks did not differentiate cells according to their VEGF-production capacity. In consideration of the large amounts of glucose present in the peritoneal cavity of PD patients, we analyzed the impact of cumulative glucose overload on VEGF levels during the whole PD period. However, glucose overload was not correlated with supernatant VEGF levels. It is necessary to involve other complementary factors in this potentially pathogenic pathway. High glucose levels are the main factor implicated in ocular angiogenesis in diabetic patients, mediated by VEGF overexpression by retinal epithelial cells (10).

In conclusion, our study demonstrates the ex vivo VEGF production by peritoneal mesothelial cells from PD patients. The great difference with respect to pleural mesothelial cells in the capacity of VEGF synthesis suggests that, possibly, factors related to PD influence this production. It is necessary to carry the research further and explain why mesothelial cells produce different amounts of VEGF.

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TABLE 2

Individual Values of Supernatant VEGF and Peritoneal Mass Transfer Coefficients (MTC) of Urea and Creatinine

Urea MTC Creatinine MTC VEGF level VEGF corrected
(mL/min) (mL/min) (pg/mL) by # cells

25.7 11.0 1448 70.2

17.5 9.9 175 9.7

19.3 13.0 662 9.3

23.5 9.2 558 7.4

27.2 10.8 780 11.6

43.3 26.2 125 1.7

21.6 8.5 184 2.9

22.9 14.2 483 5.1

35.7 14.2 1369 11.7

20.6 6.0 837 13.0

18.0 9.5 193 3.5

25.9 16.4 285 3.1

14.8 8.6 639 8.5

24.0 12.7 59 1.2

12.8 7.0 59 0.4

16.5 10.2 1747 69.9

17.5 6.9 172 1.9

10.6 2.1 127 1.3

24.0 9.2 1340 29.4

20.0 10.6 93 1.4

17.4 6.2 686 16.9

VEGR = vascular endothelial growth factor.

Normal ranges: urea MTC 18 - 22 mL/minute; creatinine MTC 7 - 11 mL/minute.

TABLE 1

Characteristics of Patients with Extreme VEGF Supernatant Levels

VEGF quartile 25	VEGF quartile 75	p Value
Supernatant VEGF (pg/mL)	92.7±33.7	1348±328 <0.01
Mesothelial cells (N)	89 250±42	663 54 513±39 006 0.21
Age (years)	61.1±8.6	55±21 0.57
Months on PD	54.1±88	21.5±19 0.4
Episodes of peritonitis	1.4±2.6	0.8±1.3 0.6
Accumulated glucose load (kg)	156.1±287	84.3±66 0.6
Creatinine MTC (mL/min)	11.7±9	10.1±3 0.7
Urea MTC (mL/min)	22.1±13	24.4±7.1 0.7
Ultrafiltration (mL/4 hr, 2.27% glucose)	650±339	540±124 0.5

VEGF = vascular endothelial growth factor; PD = peritoneal dialysis; MTC = mass transfer coefficient.

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