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Apparent successful mesothelial cell transplantation hampered by peritoneal activation

LIESBETH H.P. HEKKING, MACHTELD M. ZWEERS, EELCO D. KEUNING, BAS A.J. DRIESPRONG, DIRK R. DE WAART, ROBERT H.J. BEELEN, and JACOB VAN DEN BORN

Department of Molecular Cell Biology and Immunology, Vrije Universiteit University Medical Center, Amsterdam, The Netherlands; Department of Nephrology, Academic Medical Center, Amsterdam, The Netherlands; and Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands

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Background. Mesothelial cell transplantation has been suggested to improve mesothelial repair after surgery, recurrent peritonitis and peritoneal dialysis.

Methods. In this study we evaluated mesothelial cell transplantation during the resolution phase of experimentally thioglycollate-induced peritonitis in rats. To this end 4×10^6 DiO-labeled autologous mesothelial cells were transplanted 1 week after peritonitis induction. Peritoneal inflammation and permeability characteristics were evaluated after another week.

Results. Mesothelial cell transplantation after peritonitis resulted in incorporation of these cells in the parietal mesothelial lining, leading to an acute transient submesothelial thickening which was not seen in transplanted animals without prior peritonitis induction. Long-term functioning of these repopulated mesothelial cells leaded to peritoneal activation as evidenced by a ~twofold increase in peritoneal lymphocytes ($P < 0.01$) and omental mast cell counts ($P < 0.05$), accompanied by the induction of inflammation markers monocyte chemoattractant protein-1 (MCP-1) ($P < 0.01$) and hyaluronan ($P < 0.01$) in the transplanted peritonitis group, but not in rats with peritonitis without mesothelial cell transplantation or in control rats without mesothelial cell transplantation (all four parameters $P < 0.01$). In addition, trapping of transplanted mesothelial cells in the milky spots of omental tissue and lymphatic stomata of the diaphragm both in control and thioglycollate rats seems to increase microvascular permeability, reflected by apparent increased diffusion rates of small solutes and proteins.

Conclusion. Altogether, our data underscore the importance of controlling peritoneal (patho)physiology and function in mesothelial transplantation protocols.

Mesothelial cells cover the entire peritoneal cavity and orchestrate local inflammatory responses, regulate peritoneal microcirculation, and maintain a balance between fibrin deposition and degradation in the peritoneum [1]. The mesothelium has shown to be damaged upon peritonitis, surgery, chronic inflammation, and peritoneal dialysis. Under normal conditions, serosal healing is completed within 7 to 10 days [2]. The process of remesothelialization and the origin of new mesothelial cells remain controversial. New mesothelium is thought to be derived from mature mesothelial cells, which detach from the opposing peritoneal surface [3], migrate from the border of the injury [4], or originate from a free-floating mesothelial cell/progenitor in serosal fluid [5]. In biopsies of long-term peritoneal dialysis patients denuded areas in the mesothelial monolayer have been described [6]. Serosal injury can lead to the formation of internal scars or adhesions [7] as a consequence of an imbalance between fibrin degradation and formation by the mesothelium. Mesothelial cell transplantation is aimed to improve serosal repair.

Successful mesothelial cell transplantation has been reported in various animal models and even in a clinical study [8–11]. However, since in these studies only a single parameter was studied (adhesion formation or ultrafiltration) this conclusion might be preliminary. We therefore performed experiments to further unravel the effects of mesothelial cell transplantation during the resolution phase of experimental thioglycollate-induced peritonitis. Immunologic, morphologic, and functional parameters were evaluated. In the present study we show that successful mesothelial cell transplantation is accompanied by prolongation of the inflammatory status of the peritoneum. Our findings indicate the importance of control of peritoneal function and (patho)physiology upon therapeutic transplantation of mesothelial cells.

Key words: mesothelial cells, transplantation, peritonitis.

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METHODS

Experimental design

Experiment 1 used male 280 to 300 g Wistar rats (Harlan CPB, Zeist, The Netherlands) injected with 5 mL thioglycollate intraperitoneally. One week later, 2 mL

containing 4×10^6 DiO-labeled mesothelial cells (TM group) or 2 mL saline (TS group) was injected intraperitoneally. As control groups rats were injected ip either with DiO-labeled mesothelial cells (CM group) or saline (CS group) without prior thioglycollate. One week after mesothelial cell transplantation or saline the animals were sacrificed. A peritoneal wash was performed and peritoneal wall and omentum were excised for morphologic studies.

Experiment 2 was a parallel experiment in which a standard permeability analysis described by Zweers et al [12] was performed for 4 hours using 3.86% glucose dialysis fluid to test the permeability characteristics of the peritoneal microcirculation.

Experiment 3 was intended to study the immediate effects of mesothelial cell transplantation during the acute phase of peritonitis. Mesothelial cells were transplanted 1 day after thioglycollate injection and animals were sacrificed 2 days later. In all three experiments, six rats per group were included. The Animal Care Committee of the Vrije Universiteit of Amsterdam approved all animal experiments described.

Mesothelial cells

Primary peritoneal mesothelial cells were isolated by enzymatic digestion of the parietal peritoneal walls of adult rats and subsequently cultured as described [10, 11]. Confluent mesothelial cells (passage 4) were labeled with the lipophylic fluorescent tracer DiO (C18) (Sigma-Aldrich Chemie BV., Zwijndrecht, The Netherlands) in a final concentration of 25 μg/mL at 37°C for 16 hours culture medium. DiO belongs to the family of dialkylcarbocyanines, tracers that selectively labels the plasma membranes and do not transfer from labeled to unlabeled cells and have been used in earlier transplantation protocols (see *www.probes.com/handbook/sections/1404.html*). Labeling efficiency was always >95%. The cells were washed thoroughly, removed from the culture dish with 0.25% trypsin-0.53 mmol/L ethylenediaminetetraacetic acid (EDTA) (Gibco-BRL, Gaithersburg, MD, USA) and washed several times with Hank's balanced salt solution (HBSS) prior to intraperitoneal injection. Control injections were performed using the same volume (2 mL) HBSS.

Peritoneal effluent

Peritoneal leukocytes were collected from the peritoneal cavity by peritoneal wash with 10 mL HBSS (Gibco, BRL). Cells were counted and differentiated. In peritoneal effluent monocyte chemoattractant protein-1 (MCP-1) levels were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instruction (PharMingen, San Diego, CA, USA). Hyaluronan was determined in an ELISA-based assay essentially according to Fosang et al [13]. Interleukin (IL)-1β and IL-6 concentrations were determined by ELISA using antibody pairs (NIBSC, South Mimms, UK), and nitric oxide with Griess reagent.

Morphologic examination of the omentum and peritoneum

The omentum was dissected and spread on a glass slide for light microscopic examination. Since mast cells accumulate in the omentum upon peritoneal activation, sections were stained with toluidin blue and the density of mast cells was counted as described [14]. Specimens of peritoneal wall were frozen and sectioned at 8 μm and stained using Hoechst (10 μg/mL). Thickness of submesothelial matrix layer was evaluated using a scored eyepiece (10 measurements/animal) as described [14]. Repopulation of transplanted mesothelial cells was evaluated by fluorescence microscopy. Whole mount preparations of peritoneal wall were en face stained by conventional silver staining [11].

Statistical analysis

All data (presented as median and 25% to 75% interquartile ranges) were analyzed using the nonparametric Mann-Whitney U test. Probability values <0.05 were regarded as significant.

RESULTS

First, mesothelial cell damage by thioglycollate was visualized. To this end, thioglycollate was injected intraperitoneally and parietal peritoneal wall of the treated animals were stained with silver 1 week after the injection. As is shown in Figure 1A and B, thioglycollate focally damages the mesothelial monolayer in accordance with earlier findings [11].

Mesothelial cells were injected in control animals (CM) to evaluate homing of the mesothelial cells in animals with an intact peritoneum. As we described previously [11], no attachment of mesothelial cells was observed in the parietal peritoneum (Fig. 1C). DiO-labeled mesothelial cells were however observed in the milky spots of

Fig. 1. Thioglycollate injection damages the mesothelial cell layer of the peritoneal membrane. Representative photographs of en face silver stained parietal peritoneum of a control animal (*A*) and 1 week after a single injection with thioglycollate (*B*). The borders of the mesothelial cells as well as the damaged mesothelial cells are stained black with silver. Representative light microscopic photographs of sectioned parietal peritoneum of mesothelial cell transplanted rats with (*D*) and without (*C*) prior injection with thioglycollate. An overlay of light microscopic photograph and a fluorescence photograph is given. Transplanted mesothelial cells were a-specifically trapped into the milky spots of the omentum (*E*). DiO-labeled mesothelial cells in (D) and (E) are represented by black (D) or white (E) cells [magnification (A to D) 100×; (E) 50×].