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ORIGINAL ARTICLE

Intraperitoneal injection of cultured mesothelial cells decrease CO_2 pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model

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Abstract Mesothelial cells are involved in peritoneal repair, and they modulate adhesion formation. Intraperitoneal injection of cultured mesothelial cells can decrease adhesion formation. The importance of the amount of cells, of the culture methods or of the origin of the cells is still unknown. Female Balb/c mice were used in a CO_2 pneumoperitoneum-enhanced adhesion formation model. Mesothelial cells were obtained from Balb/c mice and cultured over a 3-week period. Following a pilot experiment (n=10) with injection of 400,000 mesothelial cells a dose–response study was performed with 400,000; 133,000; and 44,000 cells (n=40). Adhesion formation decreased after the injection of cultured mesothelial cells as evidenced in the first (proportion $p<0.046$, total adhesions: NS) and second experiment ($p<0.001$ for proportion and total adhesions). The reduction was moreover dose dependent with a half maximal effect with some 100,000 cells. Intraperitoneal injection of cultured mesothelial cells can decrease CO_2 pneumoperitoneum-enhanced adhesion formation in mice and the effect is dose dependent, the half maximum effect being obtained with some 100,000 cells, a number which is surprisingly similar to the number of cells harvested from one mouse.

Keywords Laparoscopy · Adhesions · Mesothelial cells · Mouse model

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Background

The pathophysiology of postoperative adhesions is not yet fully understood. Generally, peritoneal repair has been considered as a happening process at the traumatized areas consisting of an inflammatory reaction, exudation with fibrin deposition and appearance of macrophages, and mesothelial cells on the lesion within 24 h [1, 2]. Macrophages are believed to regulate healing through local secretion of cytokines and growth factors. They disappear within 8 days and are not incorporated into the repair [3]. When the fibrin matrix persists too long, constituting a scaffold for cellular ingrowth of fibroblasts, vessels and nerves, adhesion formation occurs.

Over the last decade, the entire peritoneal cavity has become recognized as a co-factor modulating adhesion formation at traumatized sites [4]. Identified as strongly adhesiogenic are pure CO_2 pneumoperitoneums, the addition of more than 4% oxygen to the CO_2 pneumoperitoneum, desiccation, and surgical trauma [5–8]. The mechanisms involved are believed to be mesothelial cell damage by hypoxia, by hyperoxia and reactive oxygen species, or directly by desiccation or trauma. These effects are moreover strongly attenuated at lower temperatures [9].

Prevention of adhesion formation in humans is based upon a model that considers interaction between traumatized areas. Since adhesions decreased when denuded areas were kept separated for more than 24–36 h, non-irritative

and resorbable barriers were developed or traumatized areas were kept separated by flotation agents [10].

The recognition of the peritoneal cavity as a co-factor in adhesion formation has resulted in minimizing mesothelial trauma during surgery through cellular normoxia (a partial oxygen pressure of 28 mm of Hg achieved by adding 4% of oxygen to the CO_2 pneumoperitoneum), humidification, slight cooling, and minimal tissue handling [4]. Mesothelial cells obviously play an important role in peritoneal repair. Within hours, a peritoneal defect (i.e., caused by trauma during surgery) is covered with macrophages and mesothelial cells, previously described as tissue repair cells [11]. The healing process starts from multiple spots over the defect from where mesothelial cells proliferate. Hence, small and large defects heal in the same short time (7–10 days) [12]. It is an attractive concept that mesothelial cells at the edge of a lesion and mesothelial cells of the opposing mesothelial surface have an increased dividing capacity, a phenomenon being maximal within 2 days suggest locally secreted factors [13].

The origin of these mesothelial or repair cells remains unclear. Macrophages could transform to mesothelial cells when in contact with a defect, subserosal mesothelial cells could migrate into the wound, free-floating mesothelial cells could implant while these cells could originate from bone marrow precursors [14]. Indeed, subserosal mesothelial cells are pluripotent, since they can differentiate to mesenchymal and epithelial cells. After peritoneal injury, they differentiate and start expressing cytokeratin while losing expression of vimentin [15, 16]. The exudate after injury contains a high number of fibroblast and epithelioid-like cells. These cells have low proliferative activity for at least 48 h [17]. That free-floating mesothelial cells are important is attractive since they are present at all times, their number increases 12 times in 2 to 5 days after injury [2], these cells were demonstrated to implant at the peritoneal lesion, and since extensive lavage with removal of these free-floating cells slows down peritoneal healing [18]. That mesothelial cells might originate from bone marrow precursors in blood, probably is less important since total body radiation has no effect upon peritoneal wound healing [19].

Intraperitoneally, injected exogenous mesothelial cells can implant into the injured area and reduce adhesion formation in the human by injection of cultured mesothelial cells obtained from the omentum. This confirms the observations in a staphylococcal peritonitis rabbit model [20, 21]. In a rat abrasion model, the injection of cultured mesothelial cells or mesenchymal stem cells derived from skeletal muscles of newborn rats decreased adhesion formation [22, 23]. Recently, an artificial peritoneum with collagen gel, fibroblasts, and overlying mesothelial cells significantly reduced adhesions [24]. Mesothelial cells grown from adhesions moreover are different from fibroblasts derived from normal peritoneum [25].

The concept that cultured mesothelial cells can decrease adhesion formation in an abrasion model thus seems well established. The amount of cells required, however, the culture method and the origin of the cells are still unclear [22]. Since an abrasion model is a pure surgical lesion requiring laparotomy, we therefore wanted to evaluate whether cultured mesothelial cells also affected CO_2 pneumoperitoneum-enhanced adhesion formation.

Materials and methods

Collection and culture of mesothelial cells

Mesothelial cells were obtained from 9 to 16-week-old inbred female Balb/c mice. Following anesthesia with intradermal pentobarbital (Nembutal, Sanofi Sante Animale, Brussels, Belgium; 0.06 mg/g) the abdomen was shaved, the animal was secured to the table in supine position, and the skin disinfected with a 50% alcohol solution. A 14-gauge catheter (Insyte, Vialon, Becton Dickinson) was inserted into the abdomen at the umbilicus and fixed leak proof with a Prolene 5/0 purse suture.

The cells were collected via a two-step procedure per mouse. First, 10 ml of phosphate-buffered saline pH 7.3 (Dulbecco's phosphate-buffered saline, DPBS) was injected, of which at least 8 ml could be re-aspirated. This first set of "free-floating cells" was centrifuged at 200 g for 10 min and the pellet was then suspended in culture medium RPMI 1640 (Lonza, BioWhittaker) supplemented with 15% fetal calf serum (CSL UK Ltd., Andover, UK), 4 mML-glutamine (Gibco, Paisley, UK), 0.4 mg/ml of hydrocortisone (Sigma-Aldrich, Poole, UK), and antibiotics: penicillin, 120 mg/l, amphothericine (2.5 mg/l), and gentamicin (4 mg/l) as used by Foley-Comer et al. [3]. Secondly, 10 ml of DPBS with 0.25% trypsin and 1 mM EDTA 4Na at 37°C was injected through the same catheter and the mouse was softly shaken. After 10 min, the instilled fluid was re-aspirated. Next, the abdominal cavity was washed with 5 ml culture medium; to finish, the abdominal cavity was opened and gently rinsed eight times with 2 ml culture medium. All mesothelial cells obtained via this second procedure were collected together, centrifuged for 10 min at 200 g and re-suspended in culture medium. Each time two mice had undergone the procedure, cells of those mice were pooled, keeping cells of step 1 and 2 separately. Cells were then put in an incubator at 37°C with 5% CO_2. If cells were confluent, which was normally the case after 1 week, cells were split at a ratio of 1:2, after having them detached with trypsin–EDTA. After 3 weeks, cells were detached and counted for further use (pilot, 400,000 cells; dose–response, 400,000 cells; 133,000 cells; and 44,000 cells).

Immunohistochemistry

To ascertain the growing of mesothelial cells, cells from 3-week-old cultures were seeded on slides. After 2 days of culture, the slides were fixed and endogenous peroxidase activity was blocked by 0.5% H_2O_2 in methanol. Cells were evaluated for intercellular connections and for cytokeratin staining.

Mouse model

Laparoscopic surgery

Our laparoscopic mouse model has been described and validated extensively [5]. Briefly, a 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tuttlingen, Germany) was introduced into the abdominal cavity of 9–16-week-old inbred female Balb/c mice.

CO_2 pneumoperitoneum-enhanced adhesions were induced by performing two standardized 10×1.6-mm lesions in the antimesenteric border of both right and left uterine horns and on the pelvic sidewalls with bipolar coagulation (BICAPTM, bipolar hemostasis probe, BP-5200A, 5-Fr, 200 cm; IMMED Benelux, Linkebeek, Belgium) at 20 W (Autocon 200, Karl Storz, standard coagulation mode) after which the pneumoperitoneum with pure CO_2 was maintained for exactly 60 min without desiccation (using humidified gas) at 37°C in all mice. All mice in the first experiment were operated on by RC, in the second experiment some were operated by JV who was blind for the dose of mesothelial cells injected per mouse.

Adhesions were qualitatively and quantitatively scored blindly (the investigator was not informed of the group being evaluated) 1 week later by JV during laparotomy using a stereomicroscope (Wild Heerbrugg M7A, Gais, Switzerland). The quantitative scoring system assessed the percentage of the lesions covered by adhesions as follows: adhesion proportion (%)=(sum of the length of the individual attachments/length of the lesion)×100. The qualitative scoring system assessed: extent (0, no adhesions; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% of the injured surface involved, respectively); type (0, no adhesions; 1, filmy; 2, dense; 3, capillaries present); tenacity (0, no adhesions; 1, easily fall apart; 2, require traction; 3, require sharp dissection). Total adhesion score was the sum of extent, type, and tenacity. All results represent the average of the adhesions formed at the four individual sites i.e., right and left visceral and parietal peritoneum.

Experimental design

All mice underwent four standard bipolar lesions and 1 h of CO_2 pneumoperitoneum as described above. After all laparoscopic ports had been closed, 1 ml of DPBS with or without mesothelial cells was injected intraperitoneally.

The first experiment was designed to prove that cultured mesothelial cells could prevent CO_2 pneumoperitoneum-enhanced adhesions. Mesothelial cells were collected in four mice, and cultured in eight flasks of 25 cm². Two weeks later, 2×10^6 cells were harvested. In order to investigate the effect in five mice, 400,000 cells per mouse were used. The control group, which only received DPBS, consisted of five mice. The second experiment was designed to obtain a dose–response curve of adding different amounts of cultured mouse mesothelial cells in adhesion reduction. Considering the effect of 400,000 cells from the first experiment and taking into account the number of cells available, we decided to inject per group of five mice 400,000 cells; 133,000; and 44,000 cells, i.e., a logarithmic decrease. The control group which only received DPBS consisted of five mice. The study was approved by the Institutional Review Animal Care Committee.

Statistics

Statistical analysis was performed with the SAS System (SAS Institute, Cary, NC) using the non-parametric Kruskal–Wallis test to compare individual groups. All data are presented as the mean±SE.

Findings

Culturing mesothelial cells

Culturing the harvested mesothelial cells did not provide any specific difficulty. Cultured cells were initially bipolar or multipolar in appearance, but at confluence they adopted a polygonal configuration as described by Yung et al. [26]. Viability of cells as estimated by trypan blue exclusion was over 95%. The cells had intercellular connections and were positive for cytokeratin staining confirming their mesothelial nature.

The cells from the first washing did not grow but remained viable as estimated by trypan blue exclusion after 3 weeks of culture. Their number, however, was too low for subsequent experiments.

Adhesion reduction

No mice died before the end of the experiment. In the first experiment, the injection of 400,000 cultured mesothelial cells caused a reduction of the proportion of adhesion formation from 19% to 7% ($p<0.046$), and a reduction of the total adhesion score from 3.1 (±1.74) to 1.7 (±1.68) (not significant (NS), $p<0.086$).

The second experiment confirmed that mesothelial cells can reduce CO_2 pneumoperitoneum-enhanced adhesion formation. With 44,000; 133,000; and 400,000 cells the proportion of adhesions dropped from 13.5% to 11.5% (NS), 8% ($p<0.055$), and 5% ($p<0.007$), respectively. The total adhesion score (Fig. 1) showed a similar decline being for the 44,000; 133,000; and 400,000 cells not significant, $p=0.0093$ and $p=0.0025$, respectively. When adhesions are plotted against the logarithm of the concentration of cells, the amount of cells required to decrease adhesion formation by half was estimated at 100,000 cells.

Discussion

Harvesting and growing mesothelial cells for 3 weeks is so time consuming that large scale experiments become difficult. These experiments confirm and extend the observations that cultured mesothelial cells obtained after trypsinization of the peritoneal cavity can reduce adhesion formation as described in rabbits and rats with mesothelial cells obtained from the omentum [20–22]. The effect, moreover, is not limited to pure surgical models such as the abrasion model [20, 21], but also in the CO_2 pneumoperitoneum-enhanced adhesion model. The effect is moreover dose dependent. Since we know that following a 60-min CO_2 pneumoperitoneum mesothelial cells retract thus exposing directly the basal membrane [27], we may speculate that injected mesothelial cells may fill the gaps between the retracted mesothelial cells thus attenuating the adhesion-enhancing effect of the CO_2 pneumoperitoneum by preventing the deleterious effect of the peritoneal cavity. Considering that 100,000 cells are needed for a half maximal effect, the hypothesis that the cells affect the entire peritoneal cavity seems attractive, as 100,000 cells exceed what is necessary to cover the injury which only measures 1 cm by 1.6 mm. These 100,000 cells indeed cover 10 cm^2 since a confluent culture flask of 25 cm² contains 250,000 cells. That 100,000 cells are similar to the number of cells recovered from the entire peritoneal cavity of one mouse further lends support to the hypothesis that these cells affect the entire peritoneal cavity. Whether this observation on CO_2 pneumoperitoneum-enhanced adhesions can be extended to hyperoxia, desiccation, or manipulation, enhanced adhesions are likely but remain to be established. Indeed, considering that the driving mechanisms of all these factors are micro-denuded areas all over the peritoneal cavity, the effect of cultured mesothelial cells is expected to be similar in all. A (surgical) trauma is essential to initiate adhesion formation, but these adhesions are enhanced by factors from the peritoneal cavity, the latter being quantitatively the most important. The importance of factors in the peritoneal cavity was unequivocally proven by the observation manipulation in the upper abdomen increased adhesions at the site of the surgical lesion [8]. This, moreover, was recently proven to be mediated by acute inflammation of the entire peritoneal cavity and also explains that de novo adhesions (outside the surgical lesion) were never observed.

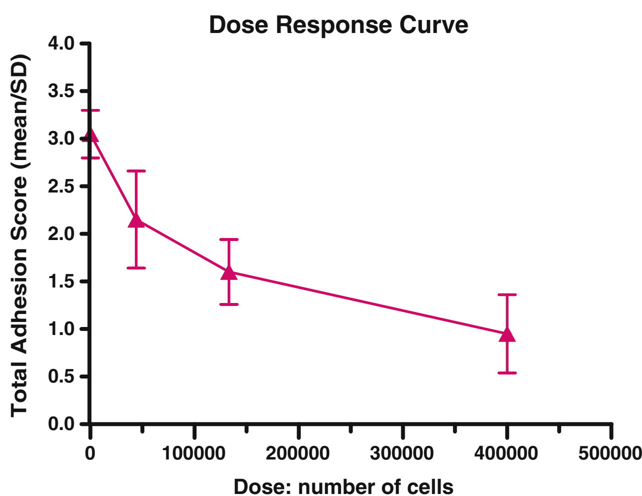

Fig. 1 Dose–response curve. *X*-axis, number of cells used per mouse

The mechanisms of adhesion reduction by cultured mesothelial cells can only be speculated upon. Whereas in pure surgical models, incorporation in the lesion as demonstrated in rats [3] seems logical, in the peritoneal cavity enhanced models incorporation in between retracted mesothelial cells of the entire peritoneal cavity cannot be excluded. Moreover, it is unclear whether the origin of the mesothelial cells and the culture conditions could be important or whether these cells actively secrete substances in the peritoneal fluid. We cannot even ascertain that the effect is specific for mesothelial cells since Alpay et al. demonstrated that also fibroblasts display different immunologic characteristics with an effect on adhesion formation [28].

It is unclear why the cells from the first washing did not grow although remaining viable. The same culture medium was used as for the detached mesothelial cells. The only difference between cells from the first washing and cells obtained after trypsinization is the fact that the former were kept for a much longer period in PBS, which indeed may damage mesothelial cells by making them lose their ability to divide (oral communication, S. Mutsaers).

The effect of mesothelial cells in reducing adhesion formation might seem less important in the pilot experiment (400,000 cells caused a 50% reduction in adhesions) than in the dose–response experiment where some 100,000 cells caused a 50% reduction in adhesions. This difference may be a consequence of the fact that cultured mesothelial cells probably stayed longer in PBS in the pilot study since our experience in handling cells was less at that moment. Also, variability in adhesion formation has to be taken into

account. Indeed, although total adhesion scores were comparable in the two control groups of both experiments (3.1) the proportion of adhesions was lower in the second experiment (13.5%) than in the first experiment (19%). Although overall very reproducible, this variability in proportion of adhesions ranging from 45% to 15% between experiments had been observed previously [29, 30] This indeed prompted us to block randomize by day while standardizing all experimental conditions as temperature, pneumoperitoneum conditions and duration, anesthesia, and experience of the surgeon as much as possible.

The clinical implications of these experiments are far reaching, whereas the use of mesothelial cells for adhesion prevention becomes attractive. Extensive lavage during and after surgery might remove besides debris and fibrin also mesothelial cells and macrophages, and the latter might be detrimental. These animal experiments combined with clinical observation at least suggest that these effects should be evaluated in detail in specific experiments. Whereas collecting and culturing mesothelial cells for adhesion prevention in the same patient is not realistic today, this method could become useful if fibroblast would be useful or if some cell lines with similar effects could be developed. In case mesothelial cells could be preserved from the peritoneal lavage during surgery as is the case with peroperative cell salvage for blood, this could be a reliable method to prevent adhesions and the detrimental effects it may cause.

Conclusions

Cultured mesothelial cells can prevent adhesion formation in mice and the effect increases with the number of cells. In Balb/c mice, the number of cells needed to reduce adhesions by 50% seems to be around 100,000. Considering that 100,000 cells cover in culture 10 cm², this suggests that the effect is mediated by incorporation in the entire peritoneal cavity and not only at the lesion site

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