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SURGICAL ENDOSCOPY
and Other Interventional Techniques

# Morphology of the rat peritoneum after carbon dioxide and helium pneumoperitoneum

## A scanning electron microscopic study

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**Abstract**
*Background:* Laparoscopic surgery for patients with cancer has been debated because of the susceptibility that laparoscopic incisions have shown for metastatic tumor growth. Structural damage of the mesothelial layer attributable to the pneumoperitoneum may facilitate intraabdominal tumor cell adhesion and growth. The influence of carbon dioxide ($CO_2$) and helium pneumoperitoneum on the morphology of the peritoneum was examined.
*Methods:* A total of 50 rats received colon carcinoma (DHB/TRb) cells intraperitoneally and $CO_2$ ($n = 25$) or helium ($n = 25$) pneumoperitoneum at 15 mmHg for 15 min. After different periods (2, 12, 24, 48, and 96 h), the rats were killed, and the peritoneum was examined by scanning electron microscopy. Control animals ($n = 5$) were without pneumoperitoneum.
*Results:* The control animals and most of the rats with pneumoperitoneum showed no peritoneal alterations. In four animals of each group, inflammatory alterations of the peritoneum such as bulging and retraction of mesothelial cells were observed at different time points. Tumor cells adherent to the peritoneum were found in a total of six animals. Peritoneal carcinomatosis, tumor nodules, or infiltration of the peritoneum by tumor cells was not observed.
*Conclusions:* The study demonstrated that the morphologic integrity of the rat peritoneum is not disturbed when $CO_2$ or helium is used for insufflation combined with the intraperitoneal injection of carcinoma cells. Pneumoperitoneum therefore probably is not the condition causing peritoneal changes that favor intraperitoneal tumor growth.

**Key words:** $CO_2$ — Helium — Pneumoperitoneum — Scanning electron microscopy — Rat — Peritoneum

Although the incidence of port-site recurrence still is unknown and seems to be rather low, tumor recurrence at this site is a dramatic complication after laparoscopic resection of malignant tumors. At the beginning of laparoscopic cancer surgery, the reported incidence of port-site metastases ranged between 1% and 21%. Recent data seem to indicate that the rate is rather low, ranging between 0% and 1.2% [12, 18, 19].

The most important mechanism in the pathogenesis of wound metastases seems to be instrumental manipulation of the tumor and mechanical spillage of tumor cells [8]. Nevertheless, there is evidence to suggest that carbon dioxide ($CO_2$) pneumoperitoneum (capnoperitoneum) creates conditions favorable to tumor growth. Carbon dioxide, shown to stimulate tumor cell growth in different animal models [9], seems to be partly responsible for metastases. Several studies have demonstrated the effects of $CO_2$ on cancer cell function that may cause changes in cell proliferation, adhesion, and invasion [3, 25].

Besides the influence of pneumoperitoneum on tumor cells, changes in oxygen ($O_2$) and $CO_2$ partial pressure, humidity, intraabdominal pressure, and temperature may affect the mesothelial layer of the peritoneum and promote cancer cell adhesion and invasion. In this context, the use of helium as an alternative insufflation gas has been discussed [16]. In experimental studies helium pneumoperitoneum led to lower rates of tumor growth and trocar-site development than $CO_2$ pneumoperitoneum [10].

The parietal peritoneum comprises a sheet of flat mesothelial cells densely covered with microvilli, the underlying basement membrane, and submesothelial connective tissue [1, 2, 11]. Its acute inflammatory

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reaction to toxic agents is characterized by bulging and separation of mesothelial cells, and by exfoliation of the cells, resulting in the exposure of submesothelial connective tissue and subsequent infiltration of the peritoneum by leukocytes and macrophages within 12 to 24 h. Several authors support the hypothesis that these changes are not agent specific, but rather represent a uniform inflammatory reaction pattern of the peritoneum [11, 13]. In experimental studies, similar alterations have been described in uremia, in the course of barium peritonitis, after exposition to starch, and after intraabdominal injection of cell-free cancerous ascites [7, 13, 14, 17, 24]. Characteristic features of bacterial peritonitis are the appearance of a fibrin network on the surface of the peritoneum and the intensive infiltration of the peritoneum by polymorph nuclear cells [13].

To determine the role of mesothelial alteration in the pathogenesis of intraabdominal tumor growth, several recent studies have examined the influence of pneumoperitoneum on the morphology of the peritoneum [6, 20, 22]. In a scanning electron microscopic study, Volz et al. [22] found that in mice, $CO_2$ pneumoperitoneum caused morphologic changes of the peritoneum such as bulging of mesothelial cells and exposure of the basal lamina, which are typical for an inflammatory reaction of the peritoneum. In a second experiment, intraabdominal cancer cell injection and $CO_2$ pneumoperitoneum led to the aforementioned alterations and to diffuse peritoneal carcinomatosis within 4 days, whereas tumor cell injection alone resulted in a nodular growth pattern of the tumor cells and did not cause alterations of the mesothelial surface [23]. According to Volz et al. [23], capnoperitoneum causes severe damage at the peritoneal membrane and thus promotes intraabdominal tumor growth. Suematsu et al. [20] reported morphologic changes in the peritoneum after $CO_2$ and helium pneumoperitoneum, which were characterized by different morphologic features, such as bulging or exfoliation of mesothelial cells. These different alteration patterns may have an influence on the intraabdominal tumor growth. Therefore, we aimed to study the impact of laparoscopy with either $CO_2$ or helium on the morphology of the peritoneum and its consequences for intraabdominal tumor growth.

## Materials and methods

### *Cell culture*

A rat colonic carcinoma cell line, DHD/K12/TRb (ECACC 90062901), was cultured in medium consisting of 50% Dulbecco's mem and 50% Ham's F-10 (Sigma, Deisenhofen, Germany) at 37°C (21% $O_2$, 5% $CO_2$), supplemented with fetal calf serum (10%, Sigma), glutamine (2 mmol/l; Gibco, Eggenstein, Germany), penicillin (100 mU/ml, Sigma), and streptomycin (100 μg/ml). Cells were grown in culture flasks (Falcon, Heidelberg, Germany).

### *Animals*

For this study, 55 male BD-IX rats (Charles River, GmbH, Sulzfeld, Germany) were acclimated to a climate- and light cycle–controlled environment (24° ± 3°C temperature, 70% ± 10% air humidity, 12 h/12 h light–dark cycle) for at least 7 days before investigations. The rats were 2 months old and weighed an average of 250 g. The animals had free access to food and water. The experiment was approved by the public animal welfare committee of the city in which the experiment was conducted. The interdisciplinary principles and guidelines for the use of animals in research, marketing, and education and the UKCCCR-Guidelines for the welfare of animals in experimental neoplasia were followed.

### *Pneumoperitoneum*

The animals were divided into two groups of 25 each. The rats were anesthetized by an intramuscular injection of rompun (2%; BayerVital, Leverbusen, Germany) and ketanest (Ursotamin; Serum-Werk, Bernburg, Germany). Next, $10^5$ carcinoma cells were injected intraperitoneally, and pneumoperitoneum was established at 15 mm Hg for 30 min (gas flow, 0.25/min). Carbon dioxide was used as the insufflation gas for group 1 and helium for group 2. The gas was insufflated with an electronic Laparoflator (Karl Storz, Germany) by the insertion of a thin needle (diameter, 1.4 mm). After 2, 12, 24, 48, and 96 h, five randomly chosen rats of each group were killed by an overdose of rompun/ketanest. Specimens 10 × 10 mm in size, each consisting of peritoneum and underlying musculature, were taken from the anterior abdominal wall. Five animals served as controls. These were anesthetized, and tumor cells were injected, but the rats did not undergo pneumoperitoneum. The control rats were killed 2 h after cancer cell injection, and specimens were taken.

### *Scanning electron microscopy*

The specimens were fixed in 2.5% glutaraldehyde in 0.1 $\mu$ of phosphate buffer, pH 7.3, for 6 h. The fixative was washed out in buffer for 2 × 15 min, postfixed in 1% osmium tetroxide in the same buffer for 120 min, washed twice in buffer for 2 × 15 min, dehydrated in a graded series of ethanol concentrations, transferred to 100% hexamethyldisilazan, and dried. Hexamethyldisilazan-dried specimens have a quality equal to critical-point-dried samples [5]. The dried tissue was mounted on a specimen holder, sputter-coated with gold (MED 020, BAL-TEC AG, Liechtenstein), and studied in a scanning electron microscope (DSM 982 Gemini; Zeiss, Göttingen, Germany).

To examine the morphology of tumor cells, sample of rat colon carcinoma cells ($10^5$ cells/ml) was treated in the same way as the peritoneal specimens. Between the single preparation steps, the cell-fixative suspension was centrifuged at 200 g.

### *Analysis of scanning electron microscopy*

If the peritoneum were affected by pneumoperitoneum, it would be expected to show alterations characteristic for an inflammatory reaction of the peritoneum after exposure to toxic agents. If these inflammatory changes depend in severity and consequences on the insufflation gas and pressure, this should become visible not only in one spot of the specimen, but rather in a generalized pattern. Therefore, we used the following algorithm: Inflammatory alterations of the peritoneum were considered: bulging of mesothelial cells, separation of mesothelial cells and denudation of the basement membrane, exfoliation of mesothelial cells, deposition of fibrin layers on the peritoneum. Every specimen was divided in to four quadrants, and each quadrant was examined separately. If one of the aforementioned alterations occurred in two or more quadrants, the sample was appointed to the diagnosis "altered." Otherwise, it was considered "intact." Scanning electron microscopy was performed at magnifications between ×200 and ×5,000 and documented digitally or by photographs.

### *Statistical analysis*

The categorical data were evaluated using Fisher's exact test. Each time point was evaluated separately, and $p$ values less than 0.05 were considered significant.

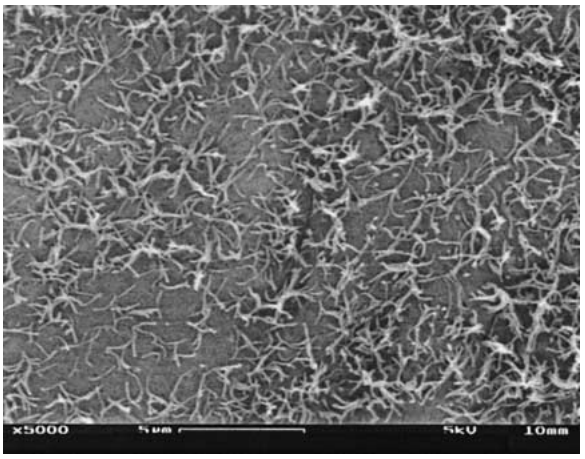


**Fig. 1.** Intact mesothelium. Magnification, ×5,000.

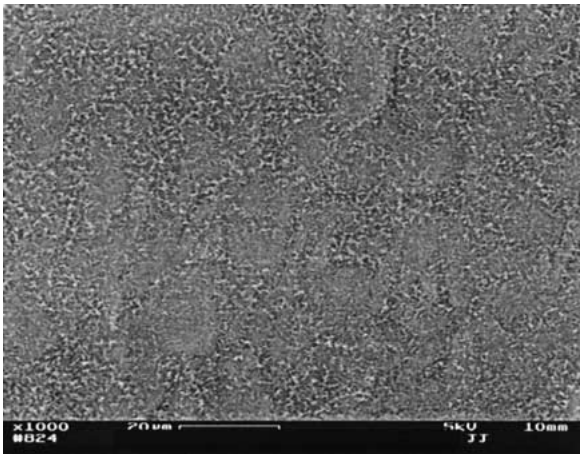


**Fig. 2.** Intact mesothelium. Magnification, ×1,000.

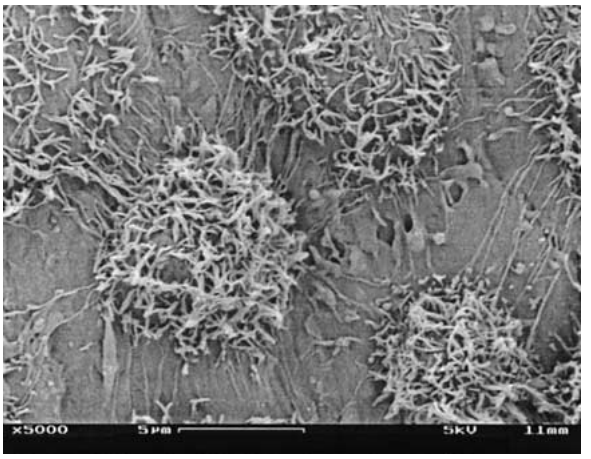


**Fig. 3.** Bulging of mesothelial cells. Magnification, ×5,000.

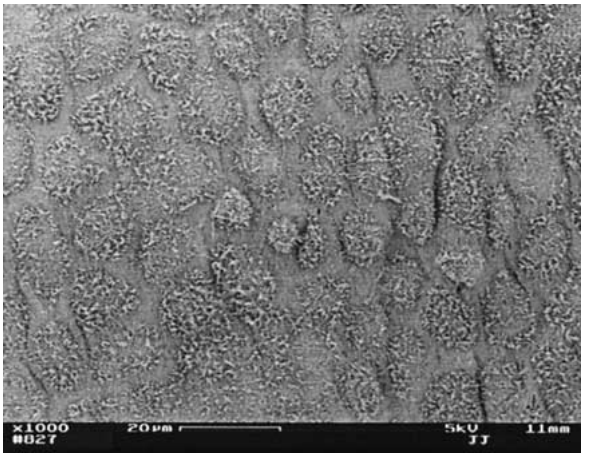


**Fig. 4.** Bulging of mesothelial cells. Magnification, ×1,000.

## Results

In the control group, the morphology of the peritoneum for all the animals appeared normal. The typical sheet of flat, microvilli-bearing polygonal mesothelial cells with diameters ranging between 12 and 27 μm was encountered (Figs. 1 and 2). No signs of inflammation were documented in the control animals.

After $CO_2$ pneumoperitoneum the peritoneum of 21 animals (84%) was considered intact. In four animals (16%) of group 1 the peritoneum showed inflammatory alterations (Figs. 3 and 4), one after 12 h, one after 24 h, and two after 96 h ($p < 0.01$), and was thus appointed to the diagnosis ''altered.'' The changes encountered were bulging or separation of mesothelial cells in two or more quadrants. After helium pneumoperitoneum, the peritoneum of 21 animals (84%) was intact. In four animals (16%), the peritoneum showed inflammatory alterations such as bulging or separation of cells, and was considered as ''altered'' (two after 2 h, one after 12 h, and one after 24 h; $p < 0.01$). Exfoliation of mesothelial cells or fibrin deposition was not found in either group.

Colon carcinoma cells were identified as round cells with a diameter of 5 to 9 μm having folds of the membrane. They occasionally revealed mitotic figures

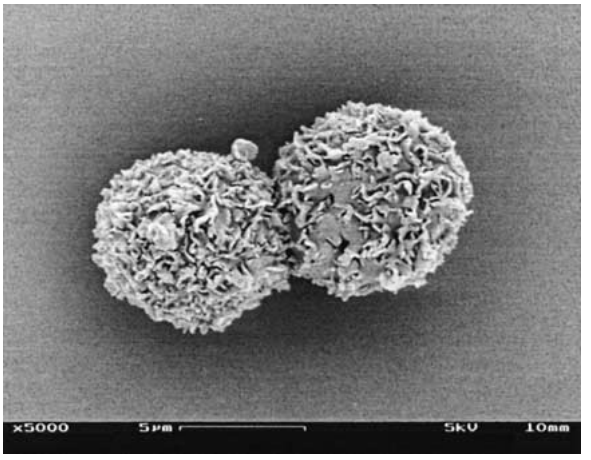


**Fig. 5.** Rat colon cancer cells. Magnification, ×5,000.

(Fig. 5). Tumor cells were found in animals treated with $CO_2$ pneumoperitoneum ($n = 6$) and helium pneumoperitoneum ($n = 3$), as well as in control animals ($n = 2$) (Fig. 6). Diffuse peritoneal carcinomatosis was not observed. There was no coincidence between the mesothelial alterations and the occurrence of tumor cells on the peritoneal surface.

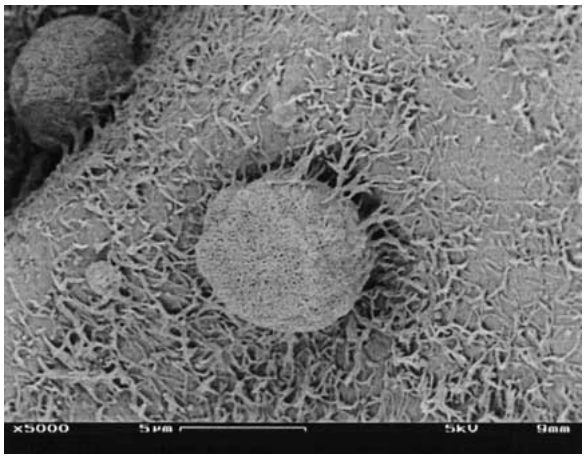


**Fig. 6.** Tumor cells in peritoneal specimen. Magnification, ×5,000.

## Discussion

Although the instrumental manipulation of the tumor and the mechanical spillage of tumor cells seem to play the major role in the development of trocar metastases, changes in the peritoneal environment and morphology during laparoscopy also may influence intraabdominal tumor growth. In general, the inflammatory reaction of the peritoneum to toxic agents is characterized by bulging and separation of mesothelial cells, and by exfoliation of the cells, resulting in the exposure of submesothelial connective tissue and subsequent infiltration of the peritoneum by leukocytes and macrophages [11, 13].

In the current study, neither $CO_2$ nor helium pneumoperitoneum led to significant inflammatory alterations of the peritoneum in the experimental animals from the different time points. The few cases in which inflammatory alterations were present occurred independently of the periods after pneumoperitoneum. Peritoneal carcinomatosis was not observed in any specimen. We therefore concluded that the experimental conditions of our study are unlikely to induce major negative changes.

However, our results do not agree with a number of observations from other recent studies. In similarly treated mice, Volz et al. [22] reported inflammatory reactions of the peritoneum such as bulging and separation of mesothelial cells, followed by regeneration processes within 96 h after $CO_2$ pneumoperitoneum. In a second series of experiments, the same authors observed that a diffuse peritoneal carcinomatosis occurred after pneumoperitoneum and concomitant intraabdominal injection of human melanoma cells, but that tumor cell application alone resulted in tumor growth of a nodular character [23]. The reason for the discrepancies between these observations and our study is not clear, but it may be related in part to the different animal species used, and to the different origin of cancer cells applied.

In another study, Suematsu et al [20] demonstrated alterations in the morphology of murine peritoneum after laparotomy and after $CO_2$ and helium pneumoperitoneum. They described bulging of mesothelial cells after $CO_2$ pneumoperitoneum, exfoliation of mesothelial cells after laparotomy, and the development of intercellular clefts after helium pneumoperitoneum. The authors believe that these distinct changes were attributable to differences between mechanical and chemical injury to the peritoneum.

At variance with this interpretation, however, it is commonly assumed that inflammatory reaction patterns are uniform and not agent specific [11, 13, 17]. Furthermore, Suematsu et al. do not explain why laparotomy, capnoperitoneum, or helium pneumoperitoneum should induce different alterations of the peritoneal morphology. The discrepancies may be related in part to the different morphologic phenotype of the visceral peritoneum studied by these authors, which principally shows stronger bulging of mesothelial cells and therefore complicates the observation of specific changes [11, 15]. Bloechle et al. [4] examined the parietal peritoneum of rats after gastric perforation-induced peritonitis and observed an aggravation of the structural peritoneum damage by the additional application of a $CO_2$ pneumoperitoneum. In agreement with the current study, however, $CO_2$ pneumoperitoneum alone did not visibly cause inflammatory changes [4]. Hazebroek et al. [6] observed the impact that humidity and temperature of $CO_2$ pneumoperitoneum had on the morphology of parietal peritoneum in rats and found retraction and bulging of mesothelial cells in all pneumoperitoneum groups. Interestingly, these authors observed in a preliminary experiment severe inflammatory changes such as exfoliation and retraction cells in an even greater degree when the gas flow was increased (personal communication).

Thus, despite the similarity of the peritoneal ultrastructure in rats and mice and their comparable tendency to display inflammatory alterations after exposure to toxic agents, it is nonetheless possible that the mentioned discrepancies are model- or technique-based. A major factor may in fact be the selected quantity of gas flow. Increased gas flow may affect peritoneal integrity by bare mechanical force, or by inducing alterations in temperature, humidity, and superficial partial gas pressure ($pO_2$, $pCO_2$). Further studies are necessary to examine the influence of gas flow on the integrity of the peritoneum.

Although scanning electron microscopy (SEM) may be considered principally as an appropriate means for evaluating peritoneal changes, criteria such as the form of mesothelial cells may be confused by site-specific differences of these cells [11, 15]. Likewise, microvilli cannot easily be used for comparison because their number and appearance may vary greatly [1, 15]. Furthermore, fixation artifacts must be taken into account. The presence of round cells and erythrocytes lying on the experimentally treated peritoneum may well be characteristic for an inflammation, but may well be attributable to contamination of the specimen with blood. Moreover, we believe that the differences between lymphocytes and macrophages, or even between tumor cells, are difficult to determine by SEM because these cells may not be distinguished reliably by mere examination of their surface structure. Shrinkage of cells

and their structural details because of fixation artifacts may need to be considered further. Virtanen et al. [21] examined the shrinkage of corneal endothelial cells caused by preparatory steps for SEM and reported a 20% reduction in diameter. This effect also had caused changes in cell morphology and size of intercellular borders, so that an analogous separation of mesothelial cells in the course of an inflammation may be misinterpreted accordingly.

Laparoscopic procedures in patients with cancer and the development of port-site metastases have raised concerns about the safety of these techniques. Although instrumental manipulation of the tumor and the mechanical spillage of tumor cells seem to be the most important events in the development of trocar metastases, changes in the peritoneal environment during laparoscopy also may influence intra- and extraabdominal tumor growth. Recent studies have documented inflammatory alterations of the peritoneum that have led to diffuse intraabdominal tumor growth after capnoperitoneum.

Contrary to those results, our study shows that the morphologic integrity of the rat peritoneum is not disturbed by a $CO_2$ or helium pneumoperitoneum. The contrary results may be based on differences in the tumor cell suspension model such as animal species or tumor cell line, or on technical features of the pneumoperitoneum besides gas and pressure such as the gas flow. Although we believe that a pneumoperitoneum does not cause morphologic changes in the peritoneal surface, we do not exclude the possibility that the alterations in peritoneal environment during laparoscopic procedures may favor intraabdominal tumor cell adhesion and growth because of its impact on physiologic cell events such as expression of adhesion molecules, secretion of cytokines, or stimulation of mitogenesis.

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