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Effect of laparoscopy versus laparotomy on circulating tumor cells using isolation by size of epithelial tumor cells

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Abstract. Lelievre L, Paterlini-Brechot P, Camatte S, Tartour E, Aggerbeck M, Vilde F, Lecuru F. Effect of laparoscopy versus laparotomy on circulating tumor cells using isolation by size of epithelial tumor cells. *Int J Gynecol Cancer* 2004;**14:**229–233.

Aim: To assess the effect of laparoscopy on circulating tumor cell (CTC) detection in case of carcinosis.

Material and methods: We compared laparoscopy versus laparotomy on tumor cell blood release in an animal model of ovarian carcinosis obtained by intraperitoneal inoculation of IGR-OV1 cells in nude rats. Animals were randomly assigned to one of the following groups: CO_2 laparoscopy (L), gasless laparoscopy (GL), midline laparotomy (ML), or general anesthesia as control (C). A 0.5 ml blood sample was taken in each case before and after experiment and tested with a novel assay, ISET (isolation by size of epithelial tumor cells), which isolates CTC by filtration on account of their size. Statistics were performed with the Fisher's and the Chi-square tests.

Results: Ten rats were included in each group. We did not find any significant difference in CTC prevalence before and after surgery (2/14 versus 3/19, respectively, $P = 1$). Similarly, the three surgical accesses were equivalent with one postexperiment detection per group: 1/5 for L, 1/7 for ML, 1/7 for GL, and 1/6 for C ($P = 0.9$).

Conclusion: This trial did not show any deleterious effect of laparoscopy on CTC when compared to laparotomy.

KEYWORDS: carcinosis, circulating tumor cells, ISET, laparoscopy, ovarian cancer.

Laparoscopy could be useful to explore and manage ovarian cancers, as it has been shown to be efficient for other pelvic cancers[1,2]. Its role on neoplastic cell spread inside the peritoneal cavity or abdominal wounds has been documented during the last 5 years. CO_2 laparoscopy enhances tumorous cell dissemination in the peritoneal cavity and risk of trocar wound metastases when compared to laparotomy or gasless laparoscopy[3,4]. On the other hand, little is known about its effects on the tumor cell release into

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the peripheral circulation. However, this topic is important since the presence of circulating epithelial tumor cells at the time of surgery has been shown to be correlated with a worse prognosis for solid tumors in clinical practice[5,6]. Concerning ovarian cancer and peritoneal carcinosis, it has recently been shown that patients with blood marrow micrometastases had a worse prognosis comparatively to patients with no micrometastasis[7]. This hematogenous spreading could occur as early as FIGO stage IC and was not correlated with peritoneal extension[7].

We recently showed that the surgical access had no influence on circulating tumor DNA in two rat models of ovarian carcinosis, one of which has been used in the present study[8]. Unfortunately, this method of detection cannot be employed to test human blood. For this new experiment, we used a novel assay called ISET (isolation by size of epithelial tumor cells), likely to be suitable for both human and animal blood[9].

Until recently, the most employed method for epithelial cells detection was reverse transcription-polymerase chain reaction (RT-PCR). ISET is an original technique which allows the isolation of circulating epithelial cells by blood filtration through a calibrated membrane. Detection and counting of the cells is made by microscopic examination of the filter membranes. Visual counting is more accurate than dosage by semiquantitative RT-PCR. This specific method has already been shown to be reliable to detect circulating cells in patients with hepatocellular carcinoma undergoing liver resection[9].

The aim of this study was to assess the effects of three different surgical accesses on the presence and quantity of tumorous cells in the peripheral blood, with the use of an ovarian carcinosis model in rats.

Materials and methods

We undertook a prospective single-blind randomized trial. To obtain our ovarian carcinosis model, an intraperitoneal inoculation of 27×10^6 IGR OV1 cells was made in female nude rats (rnu-rnu; Charles River, Sulzfdeld, Germany) at the age of 4 weeks[10]. Cells were cultured *in vitro* in RPMI 1640 medium with 10% fetal calf serum (virus and mycoplasm screened) and 5% glutamine (INSERM U190, Paris, France). After trypsine incubation, cells were washed four times in RPMI 1640 medium. Operating protocole was carried out 7 days after inoculation, as it has previously been shown to be the appropriate time interval to obtain a carcinosis with this model[11]. Procedures were performed under sterile conditions (one piece laboratory suit and overshoes, sterile gloves, facemask, sterile instruments, povidone iodine for animal skin desinfection (Bétadine, Sarget, Bordeaux, France)). General anesthesia was obtained after subcutaneous injection of ketamine (ketalar, 5 g/100 ml, 1 ml/kg) and xylasine (Rompun, 2 g/25 ml, 0.33 ml/kg). After weighing the animals, each rat was assigned at random to one of the four following groups: CO_2 laparoscopy (L) (CO_2 pneumoperitoneum, 8 mmHg pressure through an ombilical 2.1 mm Veress needle), gasless laparoscopy (GL) (abdominal wall lifted using a number 18 Foley catheter equilibrating the animal weight), midline xiphopubic laparotomy (ML), or general anesthesia as a control (C). Forty rats were included, 10 per surgical group. Each intervention lasted 1 h. A 0.5 ml peripheral blood sample was taken before and after each operation by puncture of the external jugular vein under anesthesia. Rats were sacrificed at the end of the procedure and a xiphopubic laparotomy was systematically carried out to check the presence of carcinosis, which we had determined as macroscopic tumorous granulations on the omentum and/or peritoneum.

ISET necessitates a module of filtration (licences EP513139, US5606351, JO5504405, BIOCOM Company, Les Ulis, France) and a polycarbonate 'Track-Etch-Type' membrane (Metagenex, Paris, France) with calibrated 8 μm diameter cylindrical pores. The module of filtration has 12 wells, making it possible to filter 12 individual samples in parallel. Peripheral blood was collected on buffered ethylene diaminetetraacetic acid (EDTA), then diluted to 1:10 with a filtration buffer containing 0.175% saponin, 0.2% paraformaldehyde, 0.0372% EDTA, and 0.1% bovin serum albumin, and left for 10 min at room temperature before filtration. Blood samples were processed less than 3 h after obtention if preoperative, less than 1 h if postoperative. Each sample was filtered by aspiration under vacuum through a 0.6 cm diameter surface area in the membrane. The membrane was then washed once by aspiration of phosphate-buffed saline, disassembled from the module and left to dry. Detection and counting of circulating tumor cell (CTC) was made by microscopic examination of the membranes after Haematoxylin & Eosin staining.

Before the experiment, a series of blood samples taken from 10 healthy common rats was successfully filtrated by ISET, with no epithelial cell detected. In addition, we performed feasibility tests showing that ISET could detect IGR OV1 cells mixed with rat blood *in vitro* (data not shown).

The protocol was approved by the Committee on Animal Research of the Faculté de Médecine Necker-Enfants Malades and the Veterinary Inspection of the French Agriculture Ministry.

Statistics were performed using the SPSS 7.5 statistical software (Chicago, IL) (Fisher's exact test and Pearson's Chi-square test; a P-value <0.05 being considered significant).

Results

Carcinosis was found in 100% of the cases. Filtration was successful for 44 blood samples (19 preoperative and 25 postoperative). Nineteen rats had samples filtered before and after intervention (including five controls which only underwent general anesthesia), and for six animals only results from postoperative samples were available (see Table 1).

When detection was positive, the median number of CTC per filtration spot was 2 (range: 1–3). Tumorous cells on the filter membranes were all intact, allowing their morphological analysis after Haematoxylin & Eosin staining (Figs 1 and 2). All harvested epithelial cells showed cancerous features, such as an increased size (diameter up to 50 μm) and a high nucleo-cytoplasmic ratio. We did not find any normal circulating epithelial cell, either being the result of a sample contamination by skin cells or due to the circulation of normal epithelial cells originating from any organ.

Including control animals, comparison between preoperative (or beginning of anesthesia for controls) and postoperative (or end of anesthesia) prevalences of circulating cells showed no significant difference (3/19 versus 4/25, $P=1$, Table 1). Nor was any difference found when considering operated animals only (2/14 versus 3/19, $P=1$, Table 1), or specifically studying data obtained only from the operated animals for which filtration has been possible before and after experiment ($n=14$) (2/14 versus 3/14, $P=1$, Table 1). Comparison between postoperative detection in operated rats and postexperiment detection in controls showed no difference between these two groups (3/19 versus 1/6, $P=1$, Table 1). Surgery did not enhance the amount of circulating neoplastic cells.

When studying the effect of the surgical access on cell dissemination, we found no difference between the four groups. Laparoscopy (Gasless or CO_2) did not promote tumor cell shedding more than laparotomy or general anesthesia alone did (one postexperiment detection per group: 1/5 for L, 1/7 for ML, 1/7 for GL, and 1/6 for C, $P=0.9$; Table 1).

Discussion

This study is the second we undertook that showed no deleterious effect of laparoscopy on tumor cell shedding into the peripheral circulation. Very few data are available on the effects of the pneumoperitoneum on blood dissemination. One study has shown an increased bacteremia after laparoscopy when compared to laparotomy in an animal model of peritonitis, but the difference was only observed during the first 6 h that followed the intervention, and a bacteria is far from being similar to a neoplastic cell[12]. In a prior experimental work, we analyzed the effects of laparotomy, CO_2 laparoscopy and gasless laparoscopy on circulating tumor DNA using two xenografts of ovarian carcinosis obtained by intraperitoneal inoculation of IGR OV1 or NIH OVCAR 3 human cells in nude rats[8]. Thus, blood dissemination was estimated by detection of circulating human DNA in rat blood samples by PCR. That study didn't show any adverse effect of laparoscopy, which is consistent with our present results. Since there is not any specific DNA mutation of human ovarian cancer, this method of detection cannot be employed in clinical practice; for this reason, we decided to use ISET. It is noticeable that the overall detection rate of CTC obtained with ISET after intervention, including controls after 1 h of general anesthesia (16%, 4/25) is lower than expected, as we had found circulating DNA in up to 50% of the IGR OV1 animals which underwent the previous experiment. Circulating DNA does not correlate with the exact amount of disseminating intact (and potentially aggressive) cells, as DNA can also be issued from necrotic or apoptotic cells[13]. Another explanation could be a lack of sensitivity of the method in comparison to direct PCR, which has to be confirmed.

Table 1. Circulating tumor cells detection according to surgical access

	Preoperative samples ($n=19$)		Postoperative samples ($n=25$)	
Surgical group	Detection	Total no. of samples	Detection	Total no. of samples
Laparoscopy	1	5	1	5
Midline laparotomy	1	5	1	7
Gasless laparoscopy	0	4	1	7
Controls (anesthesia only)	1	5	1	6

Preoperative (3/19) versus postoperative (4/25): $P=1$. Preoperative (2/14) versus postoperative (3/19): $P=1$ (exclusion of control rats). Preoperative only: operated rats (3/19) versus control rats (1/16): $P=1$. Postoperative only: one detection per group: $P=0.9$.

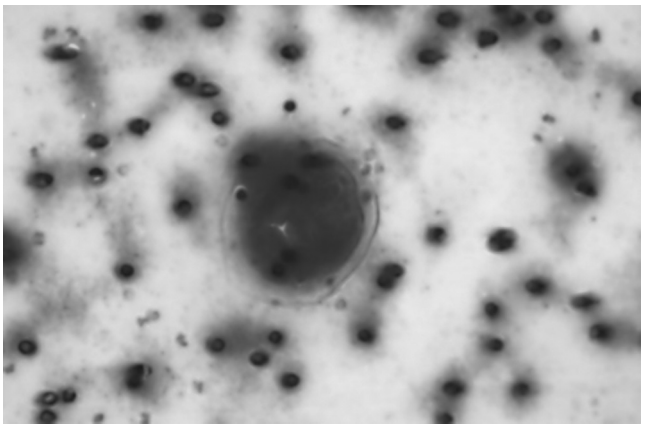

Fig. 1. Harvested IGR OV1, magnification ×40.

Sensitivity tests have been previously carried out *in vitro*[9] with the use of different cell types derived from human carcinomas (HepG2 and Hep3B from hepatocellular carcinomas, MCF-7 from breast adenocarcinoma, HeLa from cervix epithelioid carcinoma, and LNCaP from metastatic prostatic adenocarcinoma) mixed with 1 ml of peripheral blood once individually collected, to compare ISET to RT-PCR. The sensitivity threshold of ISET appeared to be close to one tumorous cell per milliliter, and RT-PCR has been found less sensitive.

Animal studies have pointed out that distant metastases are more frequently observed in operated animals than in non-operated controls[14,15]. In clinical practice, hematogenous cell shedding is promoted by diagnostic biopsies such as transrectal prostate biopsies[16] and tumor exercises like radical prostatectomy by laparotomy[17]. As for us, we did not find any significant difference between operated and control animals as well as between pre- and postoperative samples, whatever the surgical access. This lack of concordance with the literature could be explained by a minor effect of laparoscopy on systemic cell mobilization in comparison with laparotomy. The positive pressure within the peritoneal cavity associated with the effects of CO_2 on peritoneum could decrease the risk of cell seeding to the peripheral circulation. However, we did not record any effect of laparotomy either. Actually, the statistical power of this study may not be sufficient enough to prove any difference. According to a previous study that we undertook with this model[8], we should include 15 animals per group to show at least a 15% difference with α and β risks of 0.05. The study is being continued so as to achieve this goal.

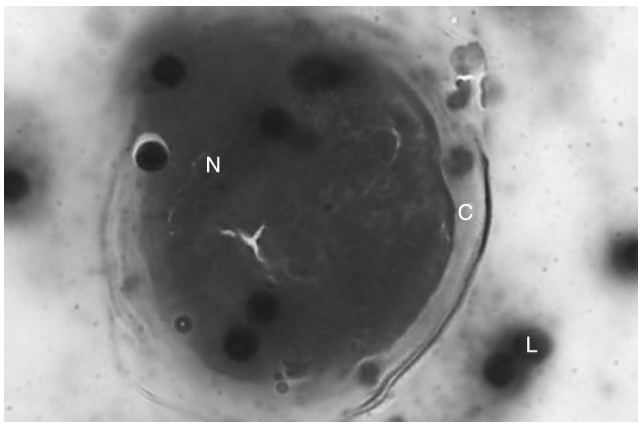

Fig. 2. Harvested IGR OV1, magnification ×100. N: Nucleus; C: Cytoplasm; L: Lymphocyte.

Furthermore, the ability of xenograft tumors to spread into the peripheral circulation might be lowered as compared to primitive human tumors.

RT-PCR, isolating in blood samples mRNAs expressed by carcinomatous cells, has been the most employed technique so far to detect hematogenous spreading[5,6]. Nevertheless chosen markers are mostly tissue-specific but not tumor-specific, such as PSA-mRNA for prostate cancer[5] cytokeratin 19 (CK19) or CEA-mRNA for colorectal cancer[6]. Consequently, false positive detection rates as high as 20% (CK19-mRNA) have been observed in disease-free patients due to marker expression by normal cells spreading from the original tissue or by epithelial cells originating from other organs[6]. This lack of specificity might constitute an explanation for the high prevalence of detection observed in clinical practice with RT-PCR, 32% (CEA-mRNA) or 73.6% (CK19-mRNA) in colorectal cancers before surgery[6] in comparison with the prevalence observed with ISET in our study (3/19 = 15%, before intervention).

Other detection methods have been described: immunobead filtration[18] measurement of telomerase activity, which is specific of neoplastic cells[19] or direct PCR if a tumor-specific mutation is known like c-Ki-ras mutations in pancreatic carcinomas[20].

ISET allows the isolation of intact epithelial cells and their microscopic examination by the cytologist leading to a precise determination of neoplastic features such as size enlargement (up to 50 μm) or high nucleo-cytoplasmic ratio (see Figs 1 and 2). In comparison with the other methods of detection, ISET allows a precise counting of circulating cells. Besides, immunohistochemistry staining can be performed providing further characterization[9]. ISET is now standardized for human blood filtration[9]. We encountered problems with several murine blood samples, 21 preoperative and 15 postoperative, due to deposits of fibrin obstructing the membrane pores making filtration impossible. This may soon be resolved by adapting

EDTA concentrations to murine blood and by reducing the time interval between blood sampling and filtration (<2 h). A dedicated ISET module within the operating room would allow us to process the preoperative samples whilst the animals are being operated on.

Finally, we assessed the feasibility of ISET to test animal blood. Accordingly, we are now able to use the same method of CTC detection in both human and animal research.

Conclusion

Our experiment did not show any difference between one surgical access and another on hematogenous cancer cell spreading. To date, there is no study available pointing out any deleterious effect of pneumoperitoneum on this dissemination, its role seems to be limited to the peritoneal cavity and trocar wounds. We intend to carry on this trial to confirm our results in a larger number of animals.

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