Local Peritonectomy Highly Attracts Free Floating Intraperitoneal Colorectal Tumour Cells in a Rat Model

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Key Words

Peritonectomy • Colorectal cancer • Local recurrence
• Rat model

Abstract

Background/Aims: Intraperitoneal free cancer cells are associated with a higher risk of recurrence and a poorer prognosis in colorectal surgery. Tumour recurrence may occur early after surgery. One potential mechanism is the ability of peritoneal lesions to attract tumour cells. **Methods:** In Wag-Rija rats, the parietal peritoneum was resected on a defined area, a corresponding control area was marked in the same rat and colorectal tumour cells (CC531) were applied into the abdomen after surgery. Tissue was harvested 6 or 9 days after surgery to evaluate intra-abdominal tumour growth. Additionally tumour cells were applied 2 weeks after peritoneal resection to investigate tumour growth in a healed area of peritonectomy. Specimens were evaluated for macroscopic tumour spread, weight of the abdominal wall and maximal tumour thickness. **Results:** Macroscopic tumour spread, weight of the abdominal wall and maximal tumour thickness were significantly increased within the area of peritonectomy after both 6 and 9 days compared to

the control area. However, only macroscopic tumour expansion was significantly increased in the healed area of peritonectomy. **Conclusion:** Peritoneal defects may play an important role in the pathogenesis of tumour implantation and might have some impact on tumour recurrence. The peritoneal damage should be kept as low as possible.

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Introduction

Recurrence and intra-abdominal tumour cell spread are major problems concerning colorectal cancer. The most frequently affected area is the former location of the primary tumour and the peritoneum [1-4]. Resection of local recurrence, even in combination with radiotherapy, is often limited by deep infiltration of surrounding tissue. Whether the peritoneum is involved, peritonectomy combined with hyperthermic intra-operative intraperitoneal chemotherapy (HIPEC) is a potentially curative treatment assuming complete cytoreduction [5, 6].

Up to now, the mechanisms for intra-abdominal or local recurrence remain unclear. An explanation might

be the biological tumour cell behaviour itself. Early recurrence after resection can be observed in patients with locally advanced, primarily or during operation perforated tumours and mucinous colorectal cancer. Elias et al described a high risk for the development of peritoneal carcinomatosis in advanced stages of colorectal carcinoma after primary resection [7]. This was confirmed by performing an elective explorative relaparotomy after one year.

Further, tumour resection either after primary colorectal cancer or after peritonectomy may result in cell leakage from the transected lymphatic tissue into the abdomen [3]. There seems to be evidence that intact basement membranes of the peritoneum are natural barriers for malignant cell implantation. Whereas damaged membranes entrap tumour cells more efficiently [3, 8]. The potential impact of peritoneal trauma by gauzes to induce metastasis of disseminated tumour cells, in a rat model, was recently described [9]. Performing colorectal surgery the mesothelium is always partially removed. Hence, abdominal and retroperitoneal lesions which potentially can attract tumour cells are therefore induced. The importance of leaking tumour cells is underlined by recent clinical data which associate the detection of intraperitoneal free cancer cells with a higher risk of recurrence and a poorer prognosis in colorectal surgery [10, 11].

Based on these findings, the aim of this experimental study was to investigate tumour inoculation in a standardized peritoneal lesion versus untouched peritoneal surface in an established rat model for peritoneal carcinomatosis.

Materials and Methods

Animals

Male pathogen-free WAG/Rija rats weighing about 250-300 g, obtained from Charles River, Sulzfeld, Germany, were used in the present study. They were fed a standard laboratory diet and tap water ad libidum. The animals were kept in individual cages. Maintenance of all experimental animals was carried out according to the guidelines of the local commission on animal protection.

Tumour cells and cell culture

The tumour cell line used was a moderately differentiated adenocarcinoma of the rat colon (tumour cell line CC531-Wag rat colon adenocarcinoma).

Tumour cells were cultured in 75cm² flasks in RPMI 1640 (Invitrogen) buffered with 20mM HEPES and 12mM NaHCO₃ and supplemented with 10% fetal bovine serum (Invitrogen),

100U/ml penicillin and 100mg/ml streptomycin. Cells were grown in a 37°C, 5% CO₂ atmosphere in a standard cell culture incubator to subconfluent monolayers.

Operation procedure

Carprofen (5mg/kg/s.c.) was given 1 hour preoperatively. Animals were then anesthetized with ketamine and xylazine (100 mg/kg/i.p.; 5mg/kg/i.p.) respectively). Thereafter the abdomen was opened by a midline incision. The peritoneum was lifted from the abdominal wall by injection of 1.5ml physiological saline under the peritoneum. Thereafter a standardized area of approximately 2x2 cm of peritoneum was thoroughly removed from the abdominal wall. The area was marked with 4 stitches using a non-resorbable thread. Within the same rat a corresponding area of the peritoneum, without surgical manipulation, was marked with 4 stitches on the contra-lateral abdominal wall, in order to have a control area within the same rat.

In the first group (group I) 2.5x10⁶ colorectal tumour cells (CC531) in 5 ml NaCl were applied into the open abdominal cavity, directly after peritonectomy. Thereafter the abdominal wall was occluded and rats received Buprenorphin (0,05 mg/kg/s.c.) for pain relief.

Rats of group I were anaesthetized for tissue harvesting 6 days (n=4) or 9 (n=7) days after tumour administration. The thorax was opened and the rats were systemically perfused with 4% phosphate buffered paraformaldehyde. The whole abdominal cavity with all abdominal organs was removed and fixed in paraformaldehyde.

In the second group (group II) (n=7) peritoneal tumour growth was investigated in an almost healed peritoneal lesions. After performing the above described surgical procedure, the rats were allowed to recover from surgery for 14 days. Thereafter the rats were anaesthetized and a mini-laparotomy was performed in order to place the tumour cells 2.5x10⁶ in 5 ml NaCl into the open abdominal cavity. Nine days after tumour cell implantation, the animals of group II, were anaesthetized for tissue harvesting in order to investigate peritoneal tumour spread.

Evaluation of local tumour spread

All animals were autopsied and the organs were macroscopically inspected. The marked area of peritonectomy and the corresponding control area were resected with a punch (diameter 2cm). All specimens were evaluated for weight, macroscopic tumour spread, and histological tumour thickness.

The resected peritoneal wall of both, the peritonectomized area and the control area was scaled. The weight is given in grams [g]. Concerning macroscopic tumour expansion of the peritonectomy area and the corresponding control area, the peritoneal side was pressed on a cover slide and the macroscopic tumour spread was marked with a water resistant black pen on a cover slide. The cover slides were scanned and TIFF files were created. The TIFF files were uploaded into scion image software (free download NIH) and a threshold for black level was set at 100.00. Accordingly marked areas of tumour manifestation (black pixels) were analyzed. The black pixels representing macroscopic tumour spread were calculated as a

percentage of the whole area which was consistent in all specimens (43500 pixels), defined by the size of the punch.

Histomorphological analyses were performed using the Leica Quantimet 550 system (Bensheim, Germany). The maximal tumour thickness within the dissected area was measured and is given in millimeter (mm).

The evaluation of local tumour spread as described above was done by blinded observers.

Results

All animals survived the surgical procedure and were alive until the end of the follow-up period. No complications were observed on daily visits and no animal developed ascites. In no animal distant pulmonary metastasis were observed. All animals developed peritoneal carcinomatosis which was confirmed by histology. In the gross overview, a substantial tumour mass could be observed by conventional light microscopy and routine H&E staining (Fig. 1a). The carcinoma infiltrates the connective tissue layer and fatty tissue and forms a solid mass which correlates to the macroscopically observed peritoneal tumour spread. The moderately differentiated adenocarcinoma induces a strong desmoplastic stroma reaction with a high cellular fibroblastic connective tissue. The adenocarcinoma forms primitive tubulus-like structures and shows mainly a trabecular growth pattern (Fig. 1b). Of note, the tumour starts infiltrating the inner abdominal muscle layer.

Fresh peritoneal lesion (Group I)

The tumour expansion in three dimensions was significantly increased in the area of peritonectomy compared to the control area. This was shown by tumour spread, the wet weight of the abdominal wall, and the histomorphological measurement.

The tumour expansion within the punched area of the abdomen was significantly increased in the area of peritonectomy compared to the control area (Fig. 2 and 3). The significantly increase accounted about 6 fold within the area of peritonectomy compared to the corresponding control area at 6 days and about 2.25 fold at 9 days, respectively (macroscopic tumour expansion [%]: control vs. area of peritonectomy at 6 days: 10.6 ± 5.5 vs. $64.3 \pm 9.5^*$; at 9 days: 39.1 ± 11.4 vs. $88.2 \pm 5.4^*$; $*p < 0.005$).

Also the weight was significantly increased in the area of peritonectomy compared to the corresponding control area, being significant after 6 days and 9 days of tumour growth (weight of the abdominal wall [mg]:

Fig. 1. (A) Overview (H&E, 25X). A solid tumour, consisting of 50% of a moderately differentiated adenocarcinoma and 50% connective tissue as desmoplastic stroma reaction. The tumour is localized in the submesothelial connective and fat tissue layer and starts infiltrating the muscle layer at the left side of the picture. (B) Detail (H&E, 200X). In detail, the adenocarcinoma shows a trabecular, in parts tubular growth pattern with atypical mitoses and apoptotic bodies. In the concomitant connective tissue, fibroblasts and scattered lymphocytes are seen.

control vs. area of peritonectomy at 6 days: 804 ± 61 vs. $1001 \pm 20^*$; at 9 days: 1348 ± 145 vs. $2168 \pm 296^*$; $*p < 0.05$).

The maximal tumour thickness was significantly increased by more than 2.6 fold within the area of peritonectomy compared to the corresponding control area at day 6 and by more than 3.8 fold at day 9 (maximal tumour thickness [mm]: control vs. area of peritonectomy at 6 days: 0.83 ± 0.14 vs. $2.19 \pm 0.26^*$; at 9 days: 1.55 ± 0.39 vs. $5.96 \pm 1.4^*$; $*p < 0.005$).

Fig. 2. Figure 2 shows the peritoneal tumour growth within 6 days. The macroscopic tumour expansion (A) was significantly increased in the area of peritonectomy compared to the corresponding control area. The weight of the abdominal wall (B) was also significantly increased in the area of peritonectomy compared to the corresponding control area. The maximal tumour height within the area of peritonectomy (C) was significantly increased compared to the corresponding control area (# $p < 0.05$, * $p < 0.005$ vs. control).

Healed area of peritonectomy (Group II)

The macroscopic tumour spread within the healed area of peritonectomy was also significantly increased compared to the corresponding control area (Fig. 4), (macroscopic tumour expansion [%]: control vs. area of peritonectomy at 9 days: 29.1 ± 7.3 vs. $69.7 \pm 8.3^*$; * $p < 0.005$).

Fig. 3. Figure 3 shows the peritoneal tumour growth within 9 days after tumour cell implantation. The macroscopic tumour expansion (A), the weight of the abdominal wall (B) and maximal tumour height (C) was significantly increased in the area of peritonectomy compared to the corresponding control area (# $p < 0.05$, * $p < 0.005$ vs. control).

However, the wet weight of the abdominal wall and the maximal tumour thickness was statistically not different in the healed area of peritonectomy compared to the corresponding control area (Fig. 4; control vs. peritonectomy; wet weight of the abdominal wall [mg]: 950 ± 66 vs. 1264 ± 166 ; maximal tumour thickness [mm]: 0.74 ± 0.34 vs. 1.37 ± 0.39 ; not significant).

Fig. 4. Figure 4 shows the peritoneal tumour growth after 9 days in the scared over peritoneal lesion. The macroscopic tumour expansion (A) was significantly increased in the area of peritonectomy compared to the corresponding control area. However, the weight of the abdominal wall (B) and maximal tumour height (C) were statistically not significantly different in the scared over peritoneal lesion compared to the corresponding control area (* $p < 0.005$ vs. control).

Comparison of the tumour growth in the fresh wound versus the healed area of peritonectomy

The macroscopic tumour expansion in the fresh peritoneal lesion was statistically not different to the healed area of peritonectomy (Fig. 5 A), however the total wet weight of the abdominal wall and the maximal tumour thickness were significantly increased in the

Fig. 5. In figure 5 tumour spread in the acute injury (grey bars) vs. tumour growth in the healed area of the peritonectomy (black bars) is shown. The macroscopic tumour expansion (A) was not different between the two groups, however, the weight of the abdominal wall (B) and maximal tumour height (C) were significantly increased in the acute peritoneal lesion (# $p < 0.05$, vs. fresh lesion).

fresh peritoneal lesion (Fig. 5 B, C); (fresh peritoneal wound vs. healed area of peritonectomy: macroscopic tumour expansion [%]: 88.2 ± 5.4 vs. 69.7 ± 8.3 ; wet weight of the abdominal wall [mg]: 2168 ± 296 vs. $1264 \pm 166^*$; maximal tumour thickness [mm]: 5.96 ± 1.4 vs. $1.37 \pm 0.39^*$; * $p < 0.05$).

Discussion

Recurrent disease in colorectal cancer is observed in about 80% of the patients. Recurrences mostly appear intra-abdominally. This pattern corresponds to findings in rectal cancer, where local recurrence occurs more often than distant metastasis [12]. In general the most common sites for tumour recurrence of primary colorectal cancers is the loco-regional region and the peritoneal surface [1-4]. It appears early after resection of locally advanced, primarily or intra-operatively perforated tumours or mucinous colorectal cancer. The mechanisms for the preferred intra-abdominal or local recurrence still remains unclear. It might be explained by the biological tumour cell behaviour itself. Further, tumour resection either after primary colorectal cancer or after peritonectomy may result in cell leakage from the transected lymphatic tissue into the abdomen [3]. Damaged membranes seem to entrap tumour cells more efficiently [3, 8]. Besides that, a recent article described the relation between trauma on the peritoneum by irritation with gauzes and tumour cell adherence in a rat model [9]. Regarding that model tumour growth was predominantly seen in traumatized areas, however a peritonectomy was not performed in these studies. In this study we were able to show for the first time that a complete local peritonectomy (fresh mesothelial lesion) and not only a local unspecific trauma is able to attract tumour cells. In colorectal surgery and especially in cytoreductive surgery (peritonectomy) and HIPEC the complete removal of the peritoneum corresponds to the clinical situation.

The data of the present study show a significantly higher tumour load in the transected area of the peritoneum compared to the corresponding control area after both 6 days and 9 days. Disseminated floating tumour cells were highly attracted by the mesothelial defect. Our results underline and fortify the hypothesis that tumour cells are entrapped by fresh lesions with acute inflammation.

Inflammatory and neoplastic disease processes of the abdominal cavity are often associated with disruption of the integrity of the mesothelium. Subsequently, peritoneal injury is accompanied with alterations of the mesothelium, leading in peritoneal healing and adhesion formation. Regarding inflammatory injury of the mesothelial cells, interleukin-1-beta seems to play an important role [13]. Van Rossen et al described the role of interleukin-1-beta and epidermal growth factor (EGF), that are significant promoting factors in tumour cell

adhesion to mesothelium in vitro [14]. Moreover neutrophils also seem to play a crucial role in the post-surgical inflammatory response and provoke enhanced tumour cell adhesion in rats [15].

It is well known that the acute inflammatory phase concerning wound healing - when growth factor and cytokine concentrations are at its maximum- is limited mainly to the first 1-3 days after injury [16, 17]. This might be a reason for the high potential of fresh lesions to attract tumour cells and enhance tumour growth. Two weeks after injury, the healing process has proceeded to the phase of maturation, while the initial inflammatory burst produced by leukocytes is over [16]. Consequently, to further evaluate the role of fresh lesions on tumour growth in comparison to a mostly healed wound we compared and analyzed the tumour growth in a healed area of peritonectomy two weeks after peritonectomy. The results of our study showed that the total tumour load is significantly lower comparing mostly healed wounds to fresh lesions. Therefore, our study is the first to show that fresh lesions attract significant more tumour cells, and thus are highly responsible for recurrence, in comparison to older lesions of the peritoneum.

Considering the clinical situation, patients with peritonectomy are usually simultaneously treated with HIPEC whereas patients with advanced local colorectal cancer receive adjuvant systemic chemotherapy. Peritonectomy combined with HIPEC offers long term survival in colorectal cancer patients suffering from peritoneal carcinomatosis (PC) [18-23]. The treatment with HIPEC has two major effects: 1.) free floating tumour cells within the abdomen are likely killed by the chemotherapeutic agent. 2.) the fresh peritoneal wound is potentially protected by the chemotherapeutic agent, since the free floating tumour cells are diminished. In regard to this, hyperthermic intra-operative chemotherapy in patients with advanced colorectal cancer with high risk of local recurrence should be discussed to become an option to overbridge the acute inflammation period. Scaringi et al used HIPEC for advanced gastric carcinoma with promising results [24]. Further adjuvant HIPEC should be evaluated with regard to peritoneal carcinomatosis [25].

In conclusion recent clinical data describe the detection of intraperitoneal free cancer cells with a higher risk of recurrence and a poorer prognosis in colorectal surgery [10, 11]. We were able to show that free floating tumour cells in the abdominal cavity are highly attracted by peritoneal lesions. This might be one important mechanism of recurrence in colorectal cancer.

HIPEC might have a great impact preventing tumour recurrence in advanced colorectal cancer and needs to be further evaluated. To avoid a surgical trauma that potentially stimulates tumour growth followed by a poor prognosis for the patient concerned, it is imperative that patients enrolled for peritonectomy should only be operated when complete cytoreduction can be achieved and peritonectomy should be limited to the tumour-affected regions. This model can be used to demonstrate the efficacy of HIPEC and other molecular agents after

peritonectomy in the future and to quantify tumour load in those areas.

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