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Original Paper

Surgical Trauma Leads to a Shorter Survival in a Murine Orthotopic Pancreatic Cancer Model

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Key Words
Immune dysfunction · Pancreatic tumour · Surgically induced immune dysfunction · Surgery · Surgical trauma

Abstract
Background: Abdominal surgery is frequently followed by immune dysfunction usually lasting for several days. This is especially important in cases with tumour diseases as an intact immune function is essential in this situation. Therefore, we analysed the outcome of tumour-bearing mice in a mouse model of surgically induced immune dysfunction (SID). ***Methods:*** In male C57BL/6 mice, a pancreatic tumour was implanted orthotopically. Following tumour implantation, the model of SID was applied. The control groups were either laparotomised or underwent no surgical procedure. The survival rate was determined by observation for >60 days. The tumour growth progress was imaged by a 7-tesla small animal MRI. ***Results:*** On day 60 after tumour implantation, the survival rate in SID mice was reduced to 41%. In the laparotomised group, 81% of mice survived, while the control group had a survival rate of 75%. These differences were significant (SID vs. control: $p < 0.02$, and SID vs. laparotomy: $p < 0.002$). The tumour volume was not influenced by the degree of surgical trauma. ***Conclusion:*** In pancreatic cancer, the SID model is ideally suited to investigate the influence of SID on this tumour entity.

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Introduction

In patients with malignant diseases, tumours often lead to severe immunosuppression. Additional dysfunctions of the immune system will further impair the prognosis of these patients. Surgery is almost always associated with a general state of immunosuppression. Abdominal surgery, in particular, is frequently followed by an immune dysfunction usually lasting for several days. Surgical procedures induce an imbalance of the immune function comparable to the course of sepsis including hypo- and hyper-inflammatory phases [1–4]. Yet, the duration of immunosuppression is not exactly known. It is believed to depend on the severity of the previous surgical trauma including duration and type of surgery.

In humans, the severity of immunosuppression is mirrored by the expression of HLA-DR (MHC class II cell surface receptor) on circulating monocytes [4, 5]. In the postoperative period, HLA-DR expression is significantly lower after major surgery, including pancreatic resections and colorectal surgery, than after minor surgery, such as cholecystectomies. In this context, the extent of surgical trauma has an important impact on surgically induced immunosuppression. This is particularly true for the duration of surgery as well as the use of minimally invasive techniques versus open surgery [6]. Procedures that last >2.5 h result in a decreased HLA-DR expression of monocytes compared to short-time surgery [7]. In particular, more complex tumour surgery, such as pancreatic or multivisceral resections, can largely extend this time frame. However, HLA-DR is expressed only by humans and therefore not a useful marker in mouse models. Nevertheless, surgery- and trauma-induced severe immunosuppression is also seen in equivalent murine models [8–11].

Despite multiple improvements in surgical and non-surgical procedures, the surgical treatment of pancreatic cancer is still associated with high morbidity rates. Unfortunately, these may lead to additional surgical interventions further impairing the immune dysfunction of pancreatic cancer patients [12].

Murine models have to include several characteristics to adequately mirror the human situation. First, the complex interactions of the host organism and tumour influencing neoangiogenesis, invasion, and formation of metastases require syngeneic models in immunocompetent hosts. Second, orthotopic tumour growth is critical for the tumour microenvironment [13, 14]. To investigate the influence of the postoperative immune dysfunction on tumour growth and tumour development, we combined an animal model of surgically induced immune dysfunction (SID) with a pancreatic carcinoma mouse model [15, 16]. We analysed survival rates and studied the tumour development employing a 7-tesla MRI. This model fulfilled all requirements to investigate the impact of immunosuppression on tumour disease.

Materials and Methods

Laboratory Animals

Male C57BL/6 mice weighing 20–23 g (obtained from Charles River Laboratories, Bad Sulzfeld, Germany) were allowed to adapt to the new surrounding for 14 days. All animal procedures were approved by the Governmental Committee (Institutional Review Board) on Animal Welfare of Mecklenburg-Vorpommern, Germany, minimizing the number of animals used. Permission was obtained from the Governmental Committee (LALLF 7221.3-1.1-026/2010).

Cell Line and Culture

The murine pancreatic adenocarcinoma cell line 6606PDA was a kind gift from Prof. David Tuveson, Cambridge, UK (current address: Cold Spring Harbor Laboratories, New York, N.Y., USA). 6606PDA cells were isolated from a pancreatic adenocarcinoma of a transgenic C57BL/6 mouse carrying a $Kras^{G12D}$ allele [17]. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml of peni-

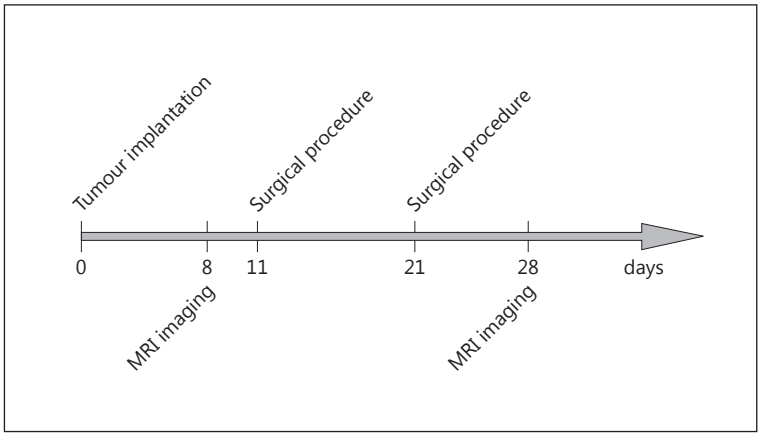


Fig. 1. Schedule of the experimental setup. On day 8 after tumour implantation, the tumour growth was analysed by 7-tesla MRI. Three days later (day 11 after tumour implantation), the mice underwent surgery. In the SID mice after laparotomy, the intestine was pressed in an antegrade direction between cotton wool swabs three times consecutively. In the laparotomy-only mice, the abdominal wall was closed 4 min after laparotomy without any abdominal organs. The control mice underwent no additional surgery. After 10 days (on day 21 after tumour implantation), the surgery was performed a second time. One week later (on day 28 after tumour implantation), the tumour progress was studied by an additional MRI scan.

cillin, and 100 µg/ml of streptomycin. Tissue culture reagents were obtained from Gibco (Invitrogen, Carlsbad, Calif., USA). Cell cultures were kept in a humidified incubator at 37°C with 5% CO_2, maintained under pathogen-free conditions and regularly tested for *Mycoplasma* species. They were consistently negative for *Mycoplasma* contamination.

Orthotopic Pancreatic Tumour Model

Pancreatic cancer cells were xenografted in mice by orthotopic injection of 6606PDA cells as described elsewhere [15]. Briefly, mice were narcotised using a combination of ketamine hydrochloride (Ketanest S®; Pfizer Pharma, Berlin, Germany) and xylometazoline hydrochloride (Rompun®; Bayer HealthCare, Berlin, Germany) at concentrations of 87 and 13 mg/kg injected intraperitoneally, respectively. 20 µl of equal volumes of PBS and matrigel (Matrigel™ Basement Membrane Matrix; BD Bioscience, San José, Calif., USA) including a total amount of 2.5×10^5 tumour cells (6606PDA) were injected into the head of the pancreas. To ensure the same injection technique for all experiments, the injection was always carried out by the same person (M.S). To decrease postsurgical pain and side effects of surgery, we subcutaneously injected buprenorphine hydrochloride at a dose of 0.1 mg/kg. Survival was monitored daily for 2 months (60 days).

Animal Model of SID

On days 11 and 21 following tumour implantation in mice, the intestine was manipulated as described before [16]. The small intestine was pressed smoothly in an antegrade direction between two sterile cotton wool swabs three times consecutively. In the laparotomy group, the abdomen was only opened and closed after 4 min without touching the intestine. Survival analyses were performed using the Kaplan-Meier procedure. All mice were checked daily for their general condition over a period of 60 days.

7-Tesla MRI

Tumour growth progress was analysed on days 8 and 28 after tumour injection. A detailed schedule of the experiment is illustrated in figure 1. Tumour-bearing mice were scanned in a high-field 7.0-T MRI scanner for small animals (ClinScan, 7.0 T, 290 mT/m gradient strength; Bruker, Ettlingen, Germany) as previously described [18]. MRI analyses were performed in a whole mouse body coil (Bruker) using a T2-TSE (turbo spin echo) sequence. For the assessment of tumour sizes, we used high-resolution T2-weighted images of the coronal as well as the axial plane. The generated images were analysed employing MIPAV (Medical Imaging Processing and Visualisation; National Institutes of Health, Bethesda, Md., USA).

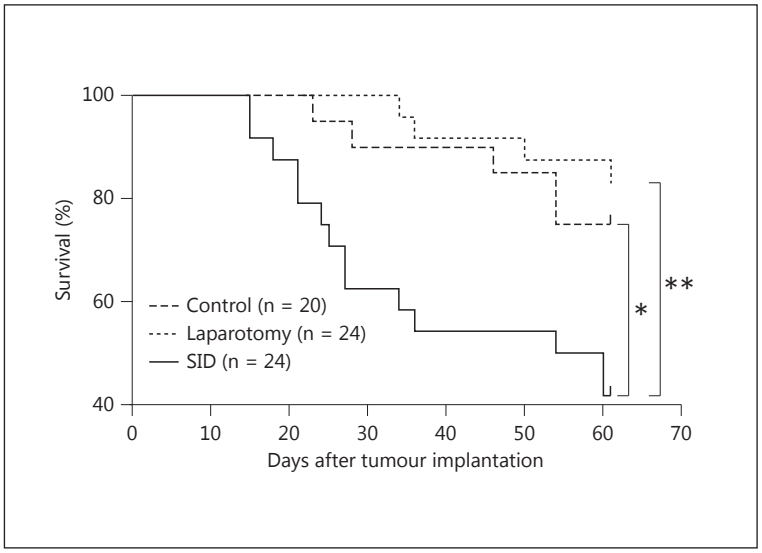


Fig. 2. Survival rates of the postoperative period. On days 11 and 21 after tumour implantation, the mice were divided into three groups: SID, laparotomy-only, or no-surgery control. Sixty days after tumour implantation, the average survival rate in the no-surgery mice was 75%, whereas the SID mice showed a survival rate of 42%. This difference was statistically significant (control: n = 20, SID: n = 24, * $p < 0.0197$). The median survival rate of the SID mice was 57 days. The laparotomy-only mice had a survival rate of 83%, which was significantly increased compared to that in the SID mice (laparotomy-only: n = 24, ** $p < 0.0015$).

Statistical Methods

Data were analysed using GraphPad Prism Version 3.02 for Windows (GraphPad Software, San Diego, Calif., USA). Statistical differences in the survival rates were assessed using the log-rank test and one-way ANOVA. A p value <0.05 was considered to be statistically significant.

Results

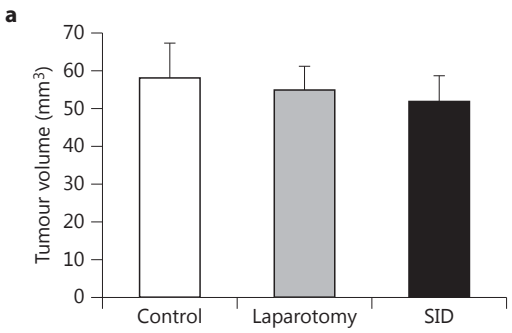
SID Worsened the Prognosis of Tumour-Bearing Mice

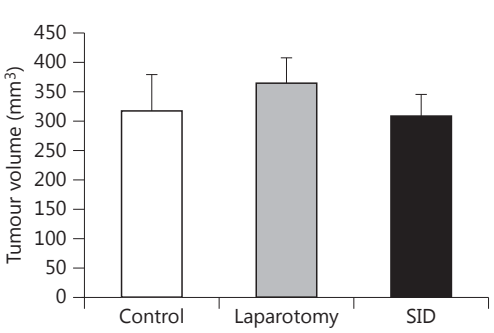
The survival rates of tumour-bearing mice were compared between those in the SID group, those in the laparotomy-only group, and those in the no-surgery control group. Sixty days following tumour implantation, the average survival rate in SID mice was 42%, with a median survival of 57 days (fig. 2). This result was statistically significant compared to the control group (75% survival rate, $p < 0.02$). In the laparotomy-only group, the survival rate was 83% and, consequently, significantly better than in SID mice ($p < 0.002$). The survival rate in control mice and laparotomy-only mice did not differ significantly ($p = 0.484$).

SID Did Not Impair Tumour Size

On day 8 following tumour injection, the average tumour volume was 55 mm^3 in all groups. No additional surgery had been performed at this time point (fig. 3a).

Four weeks after tumour implantation, SID had no effect on tumour size as measured by MRI technology. On day 28 after tumour implantation, tumours in the SID or laparotomy-only mice were not significantly different in size compared to these in the control mice: the mean (±SEM) tumour volume was 309.1 ± 35.7, 365.4 ± 42.9, and 317.9 ± 61.1 mm^3 for SID, laparotomy-only, and control mice, respectively ($p = 0.601$; fig. 3b).





	Control	Laparotomy	SID
Mice, n	9	10	10
Minimum	8.319	29.92	17.70
25% percentile	41.74	32.04	26.10
Median	52.11	56.95	64.87
75% percentile	78.26	68.96	68.86
Maximum	100.2	89.50	74.24
Mean	58.06	54.99	51.61
SD	27.36	19.33	22.06
SE	9.122	6.112	6.976
Mean 95% CI	37.03–79.10	41.17–68.82	35.83–67.39
Total	522.6	549.9	516.1

	Control	Laparotomy	SID
Mice, n	4	8	7
Minimum	172.5	178.2	198.1
25% percentile	223.6	266.7	246.3
Median	321.2	397.5	271.1
75% percentile	412.3	438.8	393.0
Maximum	457.0	538.8	472.8
Mean	317.9	365.4	309.1
SD	122.2	121.4	94.35
SE	61.11	42.93	35.66
Mean 95% CI	123.4–512.4	263.9–466.9	221.9–396.4
Total	1,272	2,923	2,164

Fig. 3. Tumour sizes depending on the type of surgery. **a** Tumour volumes were analysed by MRI on day 8 after tumour implantation. No additional surgery had been performed at this time point. The mean tumour volume was 64.87 mm^3 (range 17.70–74.24), 54.99 mm^3 (range 29.92–89.50), and 52.11 mm^3 (range 8.319–100.2) for SID mice (n = 10), laparotomy-only mice (n =10), and control mice (n = 9), respectively. **b** On day 28, the tumours of the SID mice were not significantly different from the tumours of the laparotomy-only or the control mice. The mean tumour volume was 309.1 mm^3 (range 198.1–472.8), 365.4 mm^3 (range 178.2–538.8), and 317.9 mm^3 (range 172.5–457.0) for SID mice (n = 7), laparotomy-only mice (n = 8), and control mice (n = 4), respectively; p = 0.601.

SID Did Not Induce Any Morphological Changes in Tumour Development

MRI scans did not reveal any additional morphological changes between SID mice, laparotomy-only mice, and control mice (fig. 4). Neither the zones of central necroses within the tumours nor the amounts of ascites were influenced by the type of surgery. Furthermore, the formation of metastases, including in the liver and the abdominal cavity, did not show any differences in all three groups.

Discussion

In this study, we presented a murine model to further explore postsurgical immunosuppression using a syngeneic, orthotopic murine pancreatic cancer model.

Surgical procedures are usually followed by immune dysfunction, which can last for several days. In case of sepsis in the postoperative period, surgically induced immunosup-

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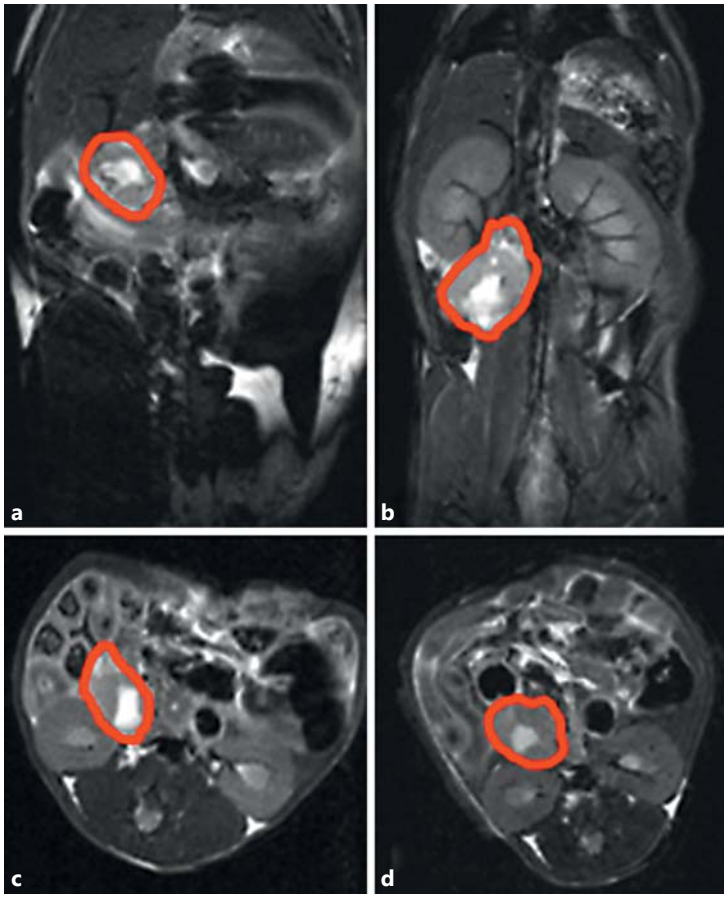

Fig. 4. MRI of a pancreatic carcinoma following surgery. Eight days following tumour implantation, tumour growth was monitored by 7-tesla MRI. **a** The T2-weighted coronal slice of a mouse with a tumour (marked by the red line) is presented. On days 11 and 21 after tumour implantation, the mice underwent surgery (SID or laparotomy only). **b** The tumours were again analysed by MRI on day 28 after tumour implantation (coronal slice). **c** An axial section of a different mouse on day 8 after tumour implantation is presented. Laparotomy was performed on days 11 and 21 after tumour implantation without touching the intestine. **d** On day 28, the tumour progress was monitored by MRI (axial section).

pression is considered to be the reason for fatalities. If peritonitis is caused by a postsurgical leakage or breakdown of an anastomosis, mortality is significantly increased compared to peritonitis induced by diverticulitis of the colon, which does not include an immunocompromising surgical procedure. Based on this accepted fact, a new classification of abdominal sepsis was suggested: type A spontaneously acquired sepsis, and type B postoperatively generated sepsis [19]. This classification has recently been revised and updated in order to include trauma-induced immune dysfunction in type B sepsis. Other entities, such as strokes, have also been added [7]. The immune status in the postoperative period depends on the previous surgical trauma and is the most important factor for the organism to manage postoperative complications.

In addition, an efficient immune system is essential to combat tumour cells in order to control tumour growth. The present study describes the impact of surgically induced immunosuppression on the outcome and the size of pancreatic tumours. Following tumour injection, the average survival rate in the no-surgery control group was 75% and did not change in the laparotomy-only group (sham operation) showing a survival rate of 83%. In contrast, the survival rate in the SID group was significantly reduced. Hence, SID significantly impaired the survival of mice. However, the tumour size was not affected by SID.

In our previous studies, we described that the immune dysfunction of SID mice is induced by intestinal manipulation and not by laparotomy alone [7, 20]. We could show that this effect correlated with the extent of the surgical trauma [16]. Furthermore, we detected elevated serum corticosterone levels in SID mice but not in mice undergoing laparotomy only [unpubl.

data]. These data also suggest that serum corticosterone levels depend on the extent of the surgical trauma [21].

In the present study, the only difference between the laparotomy group and the SID group was the immunocompromising manipulation of the intestine. We believe that the immunosuppression caused by the intestinal manipulation was the important factor inducing the significantly increased mortality in SID mice. In fact, we performed necropsies (data not shown) for possible causes of death in SID mice. However, regarding tumour volume, the amount of ascites, number and location of metastases as well as tumour cachexia, we could not find any significant differences between SID mice and control mice. In addition, we could not demonstrate any signs of macroscopic inflammation, including peritonitis or fibrin coating, for example, of the intestine. Therefore, increased mortality in SID mice is more likely to be caused by immunologic life-terminating reactions. Additional experiments addressing this issue will hopefully clarify this 'black box' in the future.

The SID procedure itself has no impact on survival. At the same time, mice do not show any signs of sepsis or inflammation at any time following SID surgery only. In our experience, the immunocompromising effects last for as long as 7 days. In our model, to maintain SID effects, we repeated the SID procedure on day 21. Interestingly, we observed a large inter-individual variability of tumour sizes on day 28. These results are in line with our previous data [18]. However, this variability as well as the limited number of research animals decreased the statistical power of the experiments to detect any association between surgical trauma and tumour growth. Finally, we could not find a correlation between tumour volumes on day 8 and survival until day 28.

As described before, the degree of intestinal trauma is the critical factor determining the extent of the postoperative immune dysfunction. Hence, intensification of the surgical trauma in the murine model results in an increased immune dysfunction as shown by the suppression of splenocyte cytokine release upon pro-inflammatory stimulation [7]. Furthermore, there are studies that describe a slight immunostimulatory effect of very limited surgical procedures [22]. Following minor surgery, the survival rate in a murine polymicrobial sepsis model (CASP) was increased compared to that in a group without previous surgical trauma [23]. Consequently, it is important to reduce the degree of surgical trauma as the most crucial factor in postoperative immunosuppression and therefore in tumour progression in general.

Conclusion

SID significantly reduces the outcome in a murine model of pancreatic cancer leaving tumour sizes unaffected. The here presented murine model has been developed to further study the impact of SID on the prognosis of pancreatic cancer.

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