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Local recurrence model of malignant pleural mesothelioma for investigation of intrapleural treatment☆

Isabelle Opitz [a], Didier Lardinois [a], Stephan Arni [a], Sven Hillinger [a], Peter Vogt [b], Bernhard Odermatt [b], Valentin Rousson [c], Walter Weder [a,*]

[a] *Division of Thoracic Surgery, University Hospital Zurich, Switzerland*
[b] *Institute of Surgical Pathology, Department Pathology, University Hospital Zurich, Switzerland*
[c] *Institute of Biostatistics, University of Zurich, Switzerland*

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Abstract

Objective: Local recurrence remains a major problem in the treatment of malignant pleural mesothelioma. The aim of the underlying study was to establish a standardised local recurrence model in rats which enables to study different intrapleural therapies. **Materials and methods:** Fifty microlitre containing 1×10^6 cells of a syngeneic rat malignant mesothelioma cell line (II-45), established from mesothelioma in Fischer 344 rats exposed to asbestos, were inoculated subpleurally via a left-sided thoracotomy. Tumour size was assessed 6 days later and the tumour nodule completely resected. Evaluation of recurrence at the resection site was performed after 10 days ($n = 6$) and 6 days ($n = 6$). The recurrent nodule was histopathologically confirmed. In a second experiment, this new recurrence model was evaluated for the effect of intrapleural therapy with different agents: 4 ml of cisplatin-solution (100 mg^2/kg BW), cisplatin combined with the fibrin-based sealant Vivostat®, 4 ml taurolidine 2%, repeated injection of 1 μg of the chemokine CCL-19 at the tumour site and 4 ml povidone-iodine in a dilution 1:10. In a control group, the chest cavity was filled with 4 ml 0.9% NaCl. The primary endpoint was the extent of tumour recurrence. **Results:** Six days after inoculation, all animals presented a standardised tumour nodule at the injection site of a mean diameter of 5.1 (±0.8) mm. Evaluation of the recurrence after 10 days showed a relapse directly at the resection site, but additional tumour nodules on the ipsi- and contralateral chest wall were found and histologically confirmed. The animals that were sacrificed 6 days after resection of the tumour nodule showed a recurrence only at the resection site with no macroscopic or microscopic evidence of other tumour. Resection of the tumour nodule combined with intrapleural application of the different agents lead to clear reduction of recurrence. The strongest effect was observed after intrapleural application of cisplatin-Vivostat® with significant decrease of the longest, widest and thickest diameter of the recurrence. **Conclusions:** With this new recurrence model for investigation of malignant pleural mesothelioma in rats, we were able to investigate new intrapleural therapies after pneumonectomy. The intrapleural application of cisplatin-Vivostat® significantly reduced the extent of local recurrence.
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Keywords: Mesothelioma; Animal model; Recurrence; Extrapleural pneumonectomy; Intrapleural; Cisplatin

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumour arising from the mesothelial lining of the pleural cavity. The incidence of MPM increased during the last 60 years, and the peak for Europe is expected between 2015 and 2020, with 250,000 predicted deaths in the next 40 years [1].

Multimodality treatment with induction chemotherapy, extrapleural pneumonectomy (EPP) and postoperative radiotherapy currently offers a median survival of 23 months [2]. But nevertheless, local recurrence is common with median time to recurrence of 16 months. Therefore, new therapeutic strategies to improve local tumour control are demanded. Intracavitary therapies such as intrapleural chemotherapy with cisplatin [3,4], immune therapy [5] and gene therapy [6] provide the advantage of easy accessibility of the large surface of the pleura and the possibility to reduce systemic side effects by increasing the desired local effect.

We were able to demonstrate sustained higher platinum concentrations in the chest wall tissue while reducing systemic levels by combination of cisplatin with a surgical sealant (Vivostat®) for intrapleural treatment in comparison to application of cisplatin alone in a rat mesothelioma model [7]. Based on the model used for this study, first described by Kucharczuk et al. [8], representing the clinical situation of invasive tumour growth of MPM after intrapleural injection of a syngeneic mesothelioma cell line, we wanted to develop a new model of local recurrence. This new model was

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* Corresponding author. Address: University Hospital Zurich, Rämistrasse 100, 8091 Zurich, Switzerland. Tel.: +41 44 255 8802; fax: +41 44 255 8805.
E-mail address: walter.weder@usz.ch (W. Weder).

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established in order to have conditions of standardised, resectable tumour and to target the problem of local recurrence in mesothelioma patients.

With the help of this model, various intrapleural therapies with chemotherapeutic agents as well as cytotoxic substances were investigated. The application of taurolidine and povidone–iodine (PVP-I) showed cytotoxic effects on human and rat mesothelioma cell lines in vitro (I. Opitz et al., in press). Both substances are used in clinical practice without important side effects: taurolidine, a derivate of the amino acid taurine, as broad spectrum antibiotic, and PVP-I as an antiseptic.

EBV-induced molecule 1 ligand chemokine (ELC/CCL19), a chemokine expressed in T-cell zones of spleen and lymph nodes, is a chemokine with proven anti-tumour efficacy by chemoattracting dendritic cells and T cells to achieve anticancer immunity. A significant reduction of tumour volume was already demonstrated after intratumoral and intranodal injections in a murine lung cancer model [9], so that we included this substance as immunomodulatory arm beside the chemotherapeutic and cytotoxic treatment groups.

The objective of the underlying study was to establish a new recurrence model for malignant pleural mesothelioma in the immune competent rat in order to prove the hypothesis that intrapleural therapy after tumour resection reduces the extent of recurrence.

2. Materials and methods

2.1. Animals and housing

Male Fischer 344 rats weighing 250–300 mg (Harlan, The Netherlands) were used. The animals were kept in the rodent facility of the University Hospital Zurich under veterinary supervision with at least 10 days acclimation period before the experiments. They had free access to standard laboratory rat chow and water, and were housed in designated rodent-storage modules in a temperature-controlled room with a 12:12 h light–dark circle. They received human care in compliance with the European Convention on Animal Care, and the study was approved by the local veterinary committee.

2.2. Cell line

A syngeneic malignant mesothelioma cell line (II-45) cultured from experimental asbestos-exposed peritoneal mesothelioma in rats was used [10]. The cell line was originally a donation from the MD Anderson Cancer Center, the University of Texas. Cells were cultured in RPMI medium supplemented with 10% fetal calf serum and a 1% solution of penicillin and streptomycin. For tumour implantation, a cell suspension of 50 μl containing 1×10^6 cells was prepared after labelling the cells with the fluorescein derivate 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma, Buchs, Switzerland) following a standard procedure [11].

2.3. Anaesthesia

After induction of anaesthesia in an isofluran chamber, oro-tracheal intubation was performed using a 16 gauge polyethylene angiocatheter which was introduced by the use of a laryngoscope. The catheter was connected to a standard rodent ventilator (Harvard Apparatus, Inc., Model 683, Germany). Ventilation was performed with mixture of oxygen and isofluran (0.5–2%, Forene, Abbott, Switzerland). A tidal volume of 10 ml/kg was used with a respiratory rate of 75/min and a PEEP of −3 cm H_2O. During surgery, the animal was placed on a warming pad and ophthalmic ointment instillated for prevention of corneal dessication. For postoperative analgesia, a subcutaneous injection of 12 mg paracetamol plus buprenorphin 0.1 mg/kg BW was performed.

2.4. Study design and surgery

2.4.1. Recurrence model

Under general anaesthesia, a tumour cell suspension of 50 μl containing 1×10^6 cells was inoculated with a thin needle under the parietal pleura via a small left-sided thoracotomy in the fifth intercostal space. The chest wall muscles were reapproximated with 4/0 Vicryl running suture, and the skin was closed with a 3/0 Vicryl running suture. After extubation, the animals were observed for 2–4 h and then returned to their cages. Six days later, thoracotomy was performed and the size of the tumour nodule at the injection site was assessed with a slide calliper and thereafter macroscopically completely resected. Evaluation of recurrence at the resection site was performed after sacrifice of the animal in the first group after 10 days ($n = 6$) and in a second group after 6 days ($n = 6$). The recurrent tumour was histopathologically confirmed.

2.4.2. Intrapleural treatment

The recurrence model was further evaluated for investigation of intrapleural treatment. Six days after tumour cell inoculation as described, the tumour nodule was resected and a left-sided pneumonectomy was performed with the help of an operating microscope (magnification 16×; Zeiss, Jena, Germany). The mediastinal pleura was dissected around the pulmonary hilum while preserving the phrenic nerve. The left main stem bronchus, the pulmonary artery and both pulmonary veins were dissected and ligated. Afterwards, a pleural abrasio was performed by scrubbing the parietal pleural with an abrader (electro-surgical tip cleaner, Surgisite®, Johnson&Johnson). This imitates the procedure of extrapleural pneumonectomy in humans, as complete dissection of the parietal pleura in rats is technically not feasible. The pleural cavity was filled with the different study substances in a randomised fashion:

1. Four millilitre of *cisplatin-solution* (100 mg/m^2): 3 mg/kg BW corresponding to 1.85 ml of a solution of 10 mg/2 ml cisplatin in a 280 g rat; 1.85 ml cisplatin was mixed with 2.15 ml 0.9% sodium chloride and filled in the chest cavity.
2. Four millilitre *cisplatin*-Vivostat®: Vivostat® (Vivolution, Denmark) is a commercialised surgical fibrin sealant. Fibrin was obtained and prepared for application as a carrier for cisplatin (100 mg/m^2) as previously described [7].
3. Four millilitre of 2% *taurolidine* (=Taurolin®, Geistlich, Wohlhusen, Switzerland).
4. Fifty microlitre of 1 μg DPBS resuspended recombinant human *CCL19*/MIP-3 beta (R&D Systems, Minneapolis,

USA) injected at the resection site, repetition of transthoracic injections on days 3 and 5.
5. Four millilitre of 0.77% *PVP-I* (=Braunol®, Braun MedicalAG, Sempach, Switzerland) in a 1:10 dilution.
6. *Control* group (4 ml 0.9% sodium chloride).

Six days after treatment, the animals were sacrificed and the whole chest wall was dissected for analysis. Two animals were treated with Vivostat® alone as a control for the effect of fibrin on the tumour.

2.5. Data analysis

The primary endpoint was the extent of tumour recurrence. The whole thoracic wall was excised and the longest and widest diameters were assessed with a tape measure. The macroscopic tumour limits were confirmed by biopsy specimens taken at both ends and analysed by fluorescence microscopy by one of the investigators blinded to the treatment (S.A.). As a third dimension, the thickness of recurrence was microscopically measured in HE section taken at the thickest diameter of the tumour nodule by a pathologist blinded to treatment (P.V.). Necrosis of the tumour was assessed qualitatively. Contralateral chest walls were histologically analysed in case of macroscopic suspicion of tumour.

The secondary endpoints were interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) present either in the cell culture supernatant of lipopolysaccharide (LPS)-activated splenocytes or directly detected in lysate of the tumour tissue at the time point of autopsy.

Splenocytes were isolated using standard procedure [11,12] co-cultivated at 1×10^6 cells/ml of mononuclear cells in complete RPMI medium or RPMI containing 5 μg/ml LPS from *E. coli*, Serotype 055:B5 (Sigma, Buchs, Switzerland) for 2 days. Supernatant was collected and frozen at −80 °C until assessment. To prepare tumour lysates, glass-on-glass pestle homogenisation of frozen biopsy specimens was performed in a ratio of 150 mg tissue/ml (w/v) in ice-cold DPBS containing a protease cocktail inhibitor (Sigma, Buchs, Switzerland). After 5 min centrifugation at 14,000 rpm, supernatant was further diluted in ELISA blocking buffer to a final 3.75 mg/100 μl and loaded in duplicate on ELISA plates. The ELISA kits used for cytokine determination were rat IL-6 (R&D Duoset Systems, Minneapolis, USA), rat TNF-α (R&D Duoset Systems, Minneapolis, USA) and rat IL-1((R&D Duoset Systems, Minneapolis, USA) according to the manufactures' protocol.

Sample size calculation was performed with the help of the Windows program called 'nQuery Advisor' Elasthoff, 1995. Statistical analysis was carried out using the software package SPSS for Windows, version 12.0 SPSS Inc. Data is given in mean and standard deviation. Data was log transformed if necessary to reach approximately normally distributed data before performing ANOVA to compare all the groups. For post hoc analysis comparisons between the groups, Bonferroni correction was applied. Differences were considered to be significant if *P*-values were smaller than 0.05.

3. Results

3.1. Establishment of the tumour model

Six days after tumour cell inoculation, all animals presented a tumour nodule at the injection site of a mean diameter of 5.1 (±0.8) mm which was macroscopically completely resected. In the first group, autopsy 10 days after resection of the tumour nodule revealed not only recurrent tumour at the resection site but also further tumour nodule spread over the ipsi- and controlateral chest wall, which was histologically confirmed. When autopsy was performed 6 days after resection of the tumour nodule, local recurrence was observed at the resection site with no macroscopic or microscopic evidence of other tumour. The histological evaluation showed a malignant pleural mesothelioma with a sarcomatous growth pattern.

3.2. Intrapleural treatment

In the second part, animals underwent tumour inoculation and resection of the tumour nodule after 6 days and a pneumonectomy was performed, followed by intrapleural treatment according to the randomisation. Perioperative death occurred in two animals. Tumour recurred at the resection site in all animals. In the untreated animals and those treated with Vivostat® alone, further tumour manifestation in the mediastinum and on the diaphragm was observed at autopsy. Comparison of the thickness, length and width of tumour recurrence between those control groups and the treatment groups showed a significant reduction in all three diameters (Table 1). The most important effect was observed after intrapleural application of cisplatin-Vivostat®, which had a significant impact on the extent of tumour recurrence (Fig. 1), as had cisplatin-solution on the thickness and the length of the tumour. The application of taurolidine lead to strong reduction of the tumour recurrence, but only the tumour length tended to be significantly influenced ($P = 0.06$). After treatment with PVP-I and CCL-19, the size of recurrence was reduced but in a not statistically significant manner (Figs. 2–4).

Table 1
Comparison of the length, width and thickness of tumour recurrence

	Control ($n = 6$)	Cisplatin-Vivostat® ($n = 7$)	Cisplatin-solution ($n = 7$)	Taurolidine ($n = 7$)	CCL-19 ($n = 7$)	PVP-I ($n = 6$)	*P*-value
Length (mean ± S.D.)	25.3 (6.3)	13.9 (3.9)	14.6 (5.7)	15.6 (5.2)	16.7 (3.7)	21.3 (8.7)	0.006
Width (mean ± S.D.)	15.5 (1.8)	4.86 (1.68)	9.4 (5)	10.3 (4.1)	11.4 (2.8)	16.5 (7.2)	0.0001
Thickness (mean ± S.D.)	5.7 (2.7)	1.61 (.077)	1.5 (0.6)	3.5 (1.6)	4.7 (2.5)	4.5 (1.4)	0.001

PVP-I: povidone-iodine; ANOVA analysis was performed.

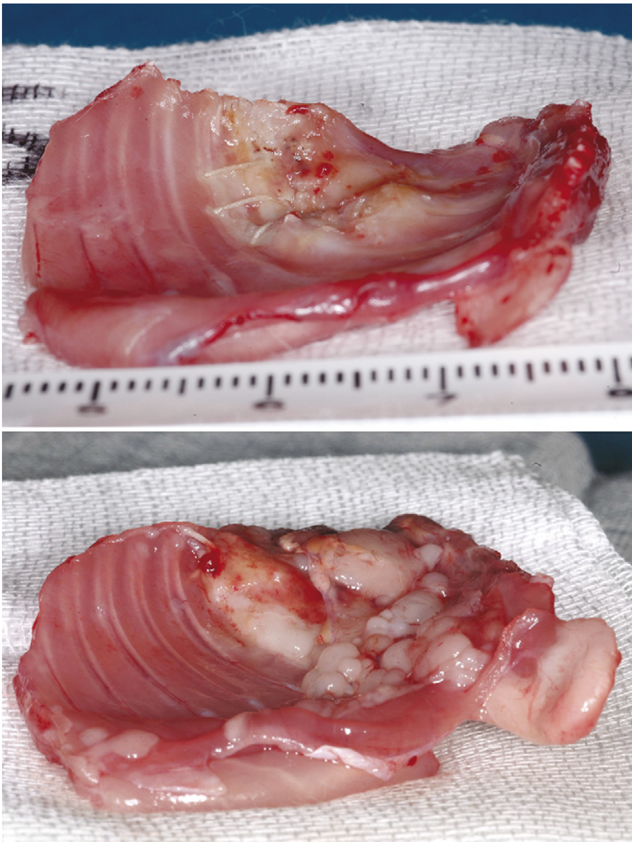

Fig. 1. Comparison of the tumour recurrence after intrapleural treatment with cisplatin-Vivostat® (upper part) to the untreated control.

Histological examination revealed necrotic formation in all the tumours of cisplatin-Vivostat® or cisplatin-solution-treated animals and in six out of seven animals treated with taurolidine. In the other groups, only 50% of the animals presented necrotic tumour (Fig. 5).

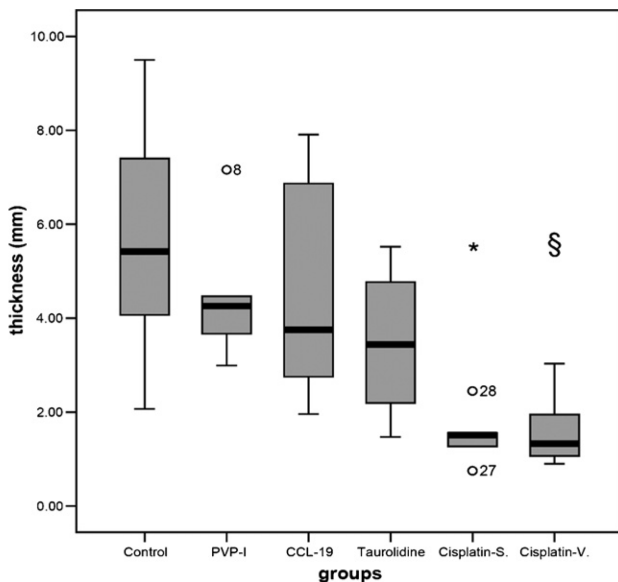

Fig. 2. Box plots of the thickness of tumour recurrence. PVP-I: povidone-iodine; Cisplatin-S.: cisplatin-solution; Cisplatin-V.: cisplatin-Vivostat®. Post hoc analysis was performed with Bonferroni corrections. $^{*}P = 0.009$; $^{\S}P = 0.005$.

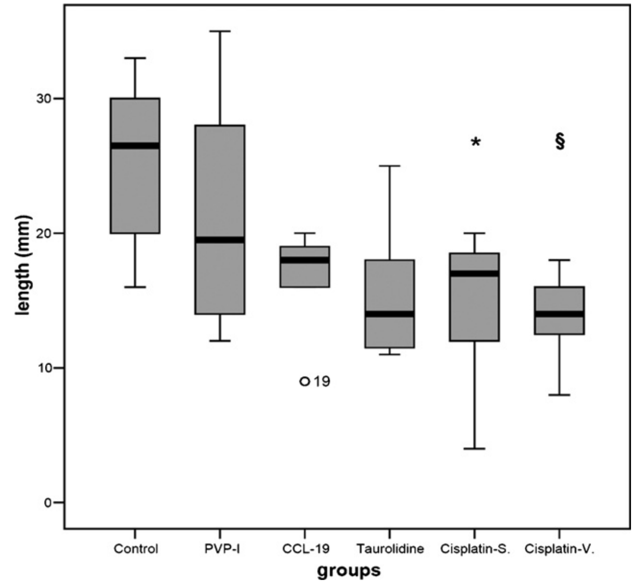

Fig. 3. Box plots of length of tumour recurrence. PVP-I: povidone-iodine; Cisplatin-S.: cisplatin-solution; Cisplatin-V.: cisplatin-Vivostat®. Post hoc analysis was performed with Bonferroni corrections. $^{*}P = 0.02$; $^{\S}P = 0.01$.

To investigate the influence of the different treatments on cytokine production, spleens were removed to obtain mononuclear cell cultures that were cultured 48 h in the presence or absence of 5 μg/ml LPS. As a control, lower concentrations of the different cytokines were detected in supernatants of mononuclear cell culture without LPS stimulation (data not shown). Cytokine determination showed significant reduction ($P = 0.03$) of the IL-6 levels in splenocyte culture supernatant after stimulation with LPS in the treatment groups in comparison to the control group and in comparison to the animals treated with Vivostat® alone (Fig. 6). A similar decrease was observed concerning the

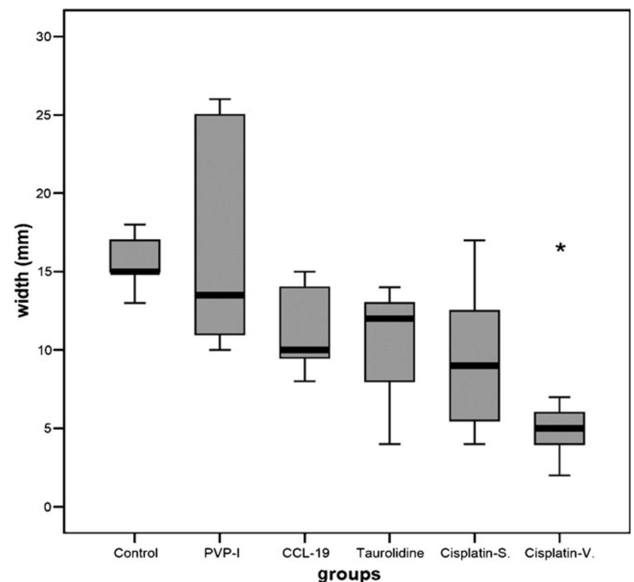

Fig. 4. Box plots of the width of tumour recurrence. PVP-I: povidone-iodine; Cisplatin-S.: cisplatin-solution; Cisplatin-V.: cisplatin-Vivostat®. Post hoc analysis was performed with Bonferroni corrections. $^{*}P = 0.001$.

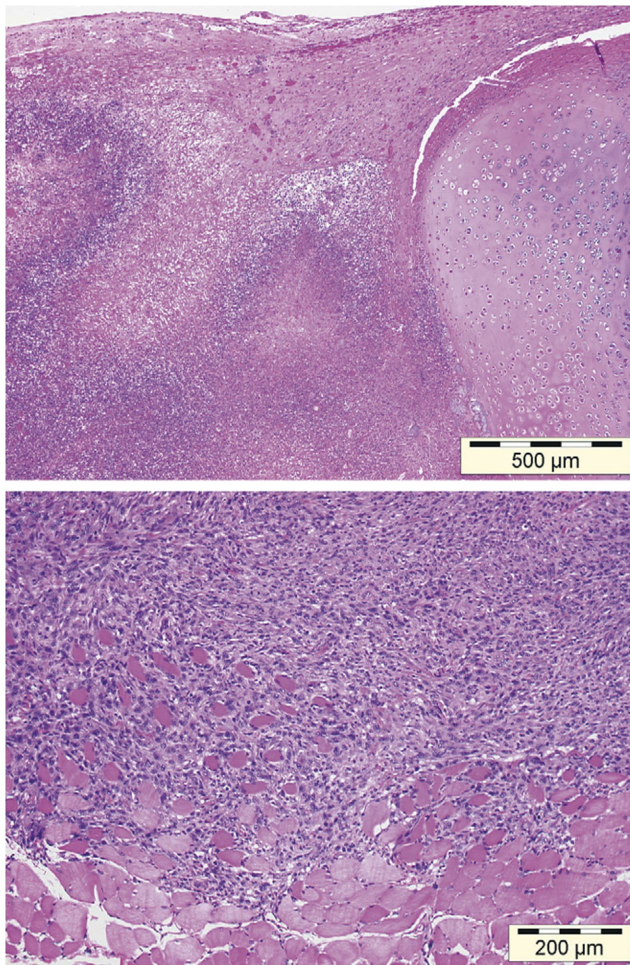

Fig. 5. Comparison of necrotic tissue at the site of recurrence after treatment with cisplatin-Vivostat® (upper part) in comparison to tumour tissue in the control group.

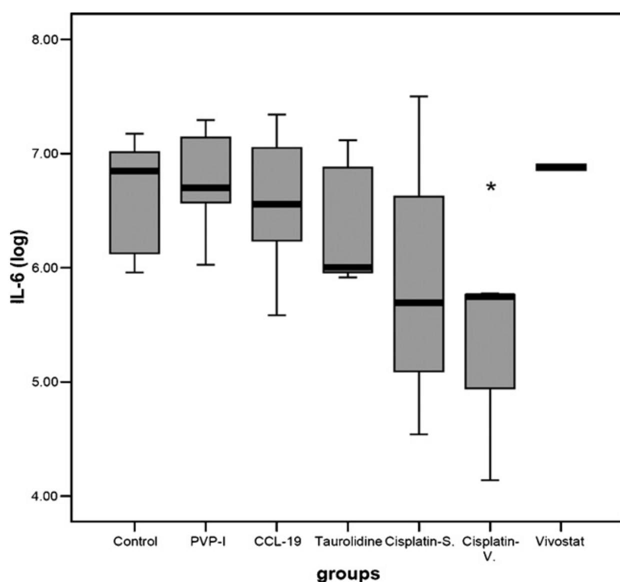

Fig. 6. IL-6 levels (pg/ml) in the splenocytes 48 h after LPS stimulation. PVP-I: povidone-iodine; Cisplatin-S.: cisplatin-solution; Cisplatin-V.: cisplatin-Vivostat®. Post hoc analysis was performed after log transforming data. $^{*}P = 0.03$.

levels of IL-1β and TNF-α in the spleens, but was not significant. Analysis of the cytokines in the tumour tissue showed no trends of changes in IL-6, IL-1β and TNF-α between the treatment groups and the control group.

4. Discussion

We were able to successfully establish a model of local recurrence of malignant pleural mesothelioma in the immune competent rat. Although several models with subcutaneous, but also orthotopic intrapleural injections of xenografted human mesothelioma cell lines exist [13,14], this does not reflect the clinical situation of patients with pleural-based disease with an intact immune system. The described model allows now to investigate therapy effects on the development of tumour recurrence in a standardised manner while reflecting the clinical situation of tumour resection in an immune competent animal.

Local intrapleural treatment is an attractive treatment option for patients with MPM, as this disease primarily remains confined to the pleural cavity, and can be administered as adjuvant strategy after EPP in order to attack minimal residual disease. The instillation of each substance tested lead to reduction of local recurrence. The application of the DNA-damaging agent cisplatin combined to the fibrin-based sealant Vivostat® caused the strongest and most significant reduction of tumour recurrence in all dimensions (length, width and thickness) and tumour was necrotic in all cases. The application of cisplatin-solution gave similar results but was not significantly better than all the other groups concerning the width of the tumour recurrence. In a previous study, we could demonstrate that platinum concentration in the chest wall remains significantly higher up to 1 week when cisplatin is combined with Vivostat® [7]. The effect of heterologous fibrin alone in the sense of an unspecific immune response against the tumour was excluded, as the behaviour of tumour recurrence was the same in the groups receiving Vivostat® alone as in the control group. Thus, the advantage of cisplatin-Vivostat® is probably due to an optimised pharmacodynamic with higher local tissue concentration whilst systemic concentrations and therefore systemic side effects are reduced. This hypothesis can be confirmed by a higher nephrotoxicity after instillation of cisplatin-solution [7]. Clinical phase I and phase II studies investigating the application of intrapleural cisplatin-chemotherapy with concentrations up to 250 mg/m^2 following pleurectomy and decortication or EPP [3,4,15] reported treatment-related complication rates up to 47% [3,15] and treatment-related deaths [16] are described. As the procedure of EPP represents a challenging procedure itself with morbidity rates up to 60% [17], reduction of systemic side effects caused by cisplatin are desired. The combination with this fibrin-based sealant may be a solution for this problem; therefore, further investigation is planned including higher concentrations of cisplatin-Vivostat®.

Taurolidine might also be an attractive therapy option as important—although only nearly significant—decrease of the recurrence size was observed after taurolidine treatment in the underlying study. As taurolidine lead in vitro to a mesothelioma cell-specific response (I. Opitz et al., in

press) and toxicity in clinical studies is remarkably low [18], it represents an interesting drug for this multimodal approach.

The effect of taurolidine has been widely described for antineoplastic treatment in ovarian cancer, lung cancer, melanoma, glioblastoma and colon cancer in vitro and in vivo [19,20]. Currently, an European multicentre study is ongoing for the investigation of adjuvant intraperitoneal irrigation with taurolidine after resection of colon cancer. Its effect is related to induction of apoptosis [20] and inhibition of growth-promoting cytokines as IL-1 or other anti-inflammatory cytokines as TNF-α [21] which have a stimulating effect on tumour growth [22]. Both of them are also described as key cytokines known to play a role in mesothelioma [23]. In our study, we observed lower levels of IL-1β and TNF-α produced by LPS-stimulated splenocytes in all treatment groups in comparison to the control group at the time point of autopsy. The same decrease occurred with IL-6 levels. Mesothelioma is reportedly an IL-6-secreting tumour [23,24]. Furthermore, IL-6 was described as a stimulating growth factor in the development of mesothelioma [25]. The significant lower levels of IL-6 after cisplatin-based treatment may reflect the destruction of not only mesothelioma cells but also of locally present immuno-regulatory cells, so that 5 days after treatment, we observed a reduced IL-6 production in the splenocytes after LPS stimulation. This might also explain the minor decrease of cytokine levels by the other substances reflecting the less aggressive mechanism of action. We were not able to detect these differences in cytokine levels in the tumour tissue. Most likely, the biopsy specimens of the tumour were too small to determine cytokine production by the tumour-infiltrating lymphocytes. Main part of the tumour had to be preserved for histological examination.

The intratumoral injection of the chemokine CCL-19 decreased the size of recurrence, but differences to the control group were not significant. Although this agent led to significant reduction of tumour volume in a murine lung cancer model [9], we were not able to reproduce this in our model. A possible explanation for this might be the use of a human form of the chemokine CCL-19 in our rat model, although it was already successfully used in other rat models [12] or an inaccurate, transthoracic technique of the repeated intratumoral injection for only 1 week. For future application, production of gene-modified dendritic cells expressing the chemokine is under investigation.

Only the application of PVP-I seemed to have practically no influence on tumour recurrence, but in this group particular problems to distinguish between tumour and neighbouring inflammatory tissue were described by the pathologist, so that data for this group may not be representative. In order to elucidate this problem, we performed immunohistochemistry. Despite detection of the CFSE labelled tumour cells in biopsy specimens by fluorescence microscopy, we failed to immunostain the CFSE dye with anti-FITC antibodies. This may be due to the fast division rate of II-45 cells and the subsequent dilution of the dye to undetectable levels for immunohistochemistry. For future projects, we have now stable transfectants of the II-45 cell line with a luciferase expressing vector allowing better distinction between tumour and surrounding tissue, as well as daily and quantitative monitoring of treatment success by bioluminescent imaging.

5. Conclusion

With this new recurrence model for investigation of MPM in rats, we were able to investigate new intrapleural therapies. The intrapleural application of cisplatin-Vivostat® significantly reduced the extent of local recurrence. Further investigation of combination treatments based on cisplatin-Vivostat® are planned.

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Appendix A. Conference discussion

Dr S. Aharinejad (*Vienna, Austria*): What was the source of the cells? Where did you get the cells from?

Dr Opitz: The cell line is a donation from the MD Anderson Cancer Center in Texas. The cell line was established by culturing cells from peritoneal mesothelioma in rats exposed to asbestos. This cell line was used for subpleural inoculation.

Dr Aharinejad: So these are rat tumour cells?

Dr Opitz: This is a syngeneic rat mesothelioma cell line.

Dr Aharinejad: And the last question, how many cells did you inject?

Dr Opitz: We injected a million cells in a volume of 50 microlitres.

Dr R. Schmid (*Bern, Switzerland*): Why didn't you do an I.V. control with the cisplatin, to see if the local treatment was really better than the systemic treatment?

Dr Opitz: This has already been investigated in clinical studies by comparing intrapleural cisplatin to intravenous cisplatin. They observed in these clinical phase I and phase II trials that the level of cisplatin was significantly higher in the pleural fluid when used as intrapleural therapy than after intravenous therapy. As we wanted to target the problem of local recurrence, we concentrated on this more effective local treatment.

Dr D. Wood (*Seattle, USA*): In that regard, you learned that not only did your model successfully result in a local recurrence but also in contralateral disease. I wonder about systemic disease, and that might direct us back to Ralph's question of systemic rather than local therapy for a model that looks like it's a timing issue of whether you catch it when it's just local versus systemic. Is your model specific enough as a local recurrence model rather than potentially a systemic recurrence model that you might look at systemic therapies, as Dr Schmid was talking about?

Dr Opitz: There is surely also the possibility to study systemic therapy in this model, but we were interested in the problem of local recurrence after tumour resection that occurs in the clinical situation earlier than distant metastases. But still it is also possible to investigate systemic therapy or combined local and systemic therapy.