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T. Zimmermann (▷) Klinik für Allgemeinund Thoraxchirurgie, Klinikstrasse 29, D-35392 Giessen, Germany Tel.: + 49-641-9944700 Fax: + 49-641-9944709 The efficacy of adjuvant cytostatic therapy after organ transplantation for malignancy: an experimental study with a combined transplantation/ tumor model

Abstract New data show that perioperative cytostatic therapy is beneficial in the case of liver transplantation for hepatic cancer. However, it has not been established clearly whether chemotherapy interferes with graft rejection. We therefore studied the interactions between tumor growth and graft rejection, especially with regard to chemotherapy, using a combined tumor/transplantation model. As a tumor model, we used the Novikoff hepatoma, a malignant hepatoma that was injected subcutaneously into the backs of rats. Heterotopic heart grafting served as the transplantation model. In a first step (a), we studied the effect of cytostatic therapy on tumor growth: tumor cells were injected, and in four groups epirubicin, cyclosporine, epirubicin + cvclosporine, and placebo were applied, in corresponding groups, transplantation was additionally performed. Tumor growth was measured and the resected tumors were examined by histology and immunohistology. In a second step (b), we studied the

effect of chemotherapy on graft rejection: transplantation was performed and the above-mentioned drugs were applied; in corresponding groups, a solid tumor was additionally induced and resected immediately before transplantation. The results of these procedures were as follows: (a) Epirubicin decreased tumor growth and diminished the volume-increasing effect of cyclosporine significantly. After transplantation, tumor growth was similar. (b) Epirubicin prolonged graft survival significantly, and the combination with cyclosporine had an augmenting effect. In the corresponding groups, graft survival was similar. In conclusions. chemotherapy diminishes the tumor-increasing effect of cyclosporine and does not interfere negatively with graft survival. It might therefore be beneficial after transplantation for malignancy.

Introduction

Liver transplantation for advanced primary liver cancer has been associated with a discouragingly high rate of tumor recurrence and poor long-term survival. Thus, considering the organ shortage, the indication for transplantation in the case of liver cancer became more and more restricted. Only in the past few years has liver transplantation for malignant hepatic tumors increasingly gained interest because of the observation that liver transplantation could result in a more favorable outcome than partial hepatectomy in cases of small hepatocellular carcinomas in cirrhotic livers [3, 7, 9]. Furthermore, new data emphasize that longterm survival after liver transplantation for hepatic carcinoma increases significantly by an adjuvant therapeutic regimen, especially neoadjuvant chemotherapy [4, 5, 14].

However, it has not been explored thoroughly whether chemotherapy has an influence on graft rejection or interferes with immunosuppressive drugs after transplantation for malignant hepatic disease. It therefore was the aim of our experimental study – using a combined transplantation/tumor model – to investigate the interactions between tumor growth and graft rejection, especially with regard to the simultaneous application of a cytostatic drug (epirubicin) and immunosuppressant (cyclosporine).

Materials and methods

For the tumor model, we used the Novikoff hepatoma, a malignant hepatic tumor induced by feeding 4-dimethylaminoazobenzene. After subcutaneous injection of 5×10^6 cells into the backs of rats, solid tumors arose. For the transplantation model, we performed heterotopic heart transplantation with the Lewis-Brown Norway rat as organ donor and Lewis rat as recipient (LEW \times BN \rightarrow LEW). Graft function was assessed by daily palpation. Rejection was defined as the complete cessation of myocardial activity. The heart transplantation model, which is well-established at our center, was used instead of a liver model because of its technical availability and convenience. The use of our heart transplantation model instead of a liver model seemed to be reasonable since the tool of our study was not the organ function itself, but rather the immunologic rejection, which can be determined precisely by the cessation of the heart beat. The heart as an organ transplant simply represented the immunologic marker in the complex interactions of immunosuppressive and cytostatic therapy.

In a first step (a), we tested the influence of epirubicin on tumor growth after transplantation. Vital hepatoma cells were injected and heterotopic heart transplantation was performed. Four groups were formed (n = 6), each receiving eithre 6 mg/kg epirubicin (i.v.), 15 mg/kg cyclosporine (i.m. for 7 days), epirubicin + cyclosporine, or placebo. In order to confirm or exclude the influence of the allogeneic graft on tumor growth, only laparotomy and clamping of the great vessels were carried out in corresponding groups, without allogeneic heart transplantation. Tumor growth was measured daily and the growth curve was recorded. On the 8th day after transplantation, the tumors were resected and examined by histology (H&E) and immunohistology (Fig. 1).

In a second step (b), we studied the influence of epirubicin on graft rejection. In order to parallel the clinical situation in which a tumor mass is resected before organ transplantation, hepatoma cells (5×10^6) were injected, and, 10 days later, the subsequent solid tumor was resected before allogeneic heart transplantation was performed. In different groups (n = 6), epirubicin (6 mg/kg), epirubicin + cyclosporine, and as controls, cyclosporine alone (low-dose; $1.5 \text{ mg/kg} \times 7 \text{ days}$), and placebo were applied. To confirm or exclude an effect of the tumor on graft rejection, only irradiated, non vital tumor cells were injected in corresponding groups. The heart beat was monitored and the time of graft rejection was determined (Fig. 2).

Drug doses were chosen in accordance with a prestudy evaluation. Epirubicin was used as cytostatic drug because it is given frequently in the treatment of human hepatic carcinoma. In contrast to the situation concerning humans, for which 2 mg/kg is a suitable dosage, 6 mg/kg epirubicin proved to be effective on tumor growth in our experimental protocol, but did not interfere negatively with



Fig.1 Experimental design: the influence of epirubicin on tumor growth after transplantation. Hepatoma cells were injected and heterotopic heart transplantation was performed. After the operation, epirubicin (or cyclosporine, epirubicin + cyclosporine, or placebo, respectively) was applied. (*) For control, heart transplantation was omitted. Tumor volume was measured and after 8 days the tumor was examined by histology



Fig.2 Experimental design: the influence of epirubicin on graft rejection. A heterotopic heart transplantation was performed and epirubicin (or cyclosporine, epirubicin + cyclosporine, or placebo, respectively) was applied. Ten days before, tumor cells had been injected; the subsequent tumor was resected immediately before transplantation. (*) For control, nonvital tumor cells were injected and consequently no tumor had to be resected

the condition of the animals. In our first study design (a), 15 mg/ kg cyclosporine was used because it results in an unlimited survival of the grafted hearts. In the second protocol (b), studying the influence of epirubicin on graft rejection, 1.5 mg/kg cyclosporine was taken since this dosage prolonged graft survival significantly without suppressing the effect of the cytostatic drug.

When the tumor growth was measured and the heart function was monitored, the examined animals of the different groups were blinded for the investigator.

Tumor growth curves were compared by means of the Wilcoxon test and the graft survival rates by the log rank test.

The German Law on the Protection of Animals was followed. This work was authorized by the Regierungspräsidium Giessen 17b-19c 20-15 (1) Gi 20/14 2/91 and 17a-19c 20-15 (1) Gi 20/ 14-2/93.





Results

(a) Cyclosporine increased tumor growth significantly, but this effect was counterbalanced by the addition of epirubicin: when cyclosporine was applied, the tumor volume increased significantly in comparison to the placebo-treated groups both after organ transplantation $(6.24 \text{ vs } 3.34 \text{ cm}^3; P = 0.035)$, as well as in the corresponding groups that did not undergo heart grafting (6.37 vs 3.34 cm³; P = 0.002). But this cyclosporine-induced effect was reversed, when epirubicin was added: if only epirubicin was applied, tumor growth decreased markedly, even though significance was failed. Eight days after tumor cell injection, the tumor volume was 2.37 cm³ after the application of epirubicin and 3.34 cm³ (P = 0.24) after the application of placebo in the transplant groups and 2.44 vs 3.34 cm³ (P = 0.09), respectively, when heart grafting was not performed. But, as mentioned above, epirubicin was able to diminish the volume-increasing effect of cyclosporine; the tumor volume after the application of the combination of epirubicin and cyclosporine was nearly identical to that of epirubicin alone, both after transplantation (2.37 vs 2.50 cm³; P = 0.84) and when allogeneic hearts were not grafted (2.44 vs 2.40 cm³; P = 0.15) (Fig. 3).

When the corresponding groups, i.e., those undergoing and those not undergoing transplantation, were compared, no difference could be found, thus excluding an influence of the allogeneic graft on tumor growth. Histological examination of the resected tumors again revealed no difference between the transplant and nontransplant groups. H&E-stained sections of the resected tumors showed a vital solid tumor when placebo was administered. When cyclosporine was added, the vital tumor cells on the border widely infiltrated the adjacent muscle, and in the large central tumor a necrosis could be found. The application of epirubicin was followed by extensive regressive alterations. There was nearly no difference between the sections the tumors treated with epirubicin + cyclosporine and those treated with epirubicin alone. Immunohistological examination showed a markedly reduced expression of T CD8 cells (monoclonal antibody Ox-8) when administering cyclosporine. In the epirubicin-treated tumors, a marked reduction of the monoclonal antibody W3–25, expressing T CD4 cells, could be found.

(b) Epirubicin prolonged graft survival significantly. after resection of a previously solid tumor as well as in the corresponding group without the induced tumor. Graft survival was 12.8 days after epirubicin and 8.3 days after placebo (P = 0.017) in the tumor group, and 13.3 vs 7.2 days (P = 0.019) in the group without tumor. Epirubicin had a similar effect on graft rejection as low-dose cyclosporine, which equally extented graft survival significantly to 17.7 (P = 0.011) and 15.8 (P = 0.019) days, respectively. The combination of the cytostatic and the immunosuppressive drug even had an augmenting effect: graft survival was 21.5 days with the application of epirubicin and cyclosporine after tumor resection, and 20.2 days in the group without tumor. When the corresponding groups with and without tumor were compared, no difference could be seen; the histoincompatible heart was rejected after nearly the same period, thus excluding an effect of tumors resected before transplantation on graft rejection (Fig. 4).

Fig.4a, b The influence of epirubicin and/or cyclosporine on graft rejection. a Groups in which vital tumor cells had been injected 10 days before heart transplantation, b groups without vital tumor cells



Discussion

In the early days of liver transplantation, primary malignant liver disease became a frequent indication because of the favorable short-term outcome with less postoperative complications than when transplantation was performed for advanced benign disease. Moreover, total hepatectomy and liver replacement have been regarded as a logical extension of partial hepatectomy. However, the long-term results of liver transplantation carried out for large malignant liver tumors that could not be removed by conventional techniques were discouraging because of the high rate of local tumor recurrence and distant metastases. The number of liver transplantations performed for malignancy has therefore decreased continuously in the following decades, down to a share of about 15% in 1990, whereas the total number of liver transplantations has increased over the same period [2]. In the course of the last few years, however, transplantation regained importance in the surgical treatment of hepatocellular carcinoma: new data suggest that the best indications are small tumors in cirrhotic livers. In the treatment of these small tumors, better results can be achieved by allogeneic transplantation than with conventional resection [3, 7, 9].

Furthermore, these results might be improved by an adjuvant cytostatic therapy. In a randomized prospective study by the Pittsburgh group, 1-year disease-free survival could be increased from 36% to 82% by an adjuvant regimen [4]. Based upon this experience, they recommended to restrict liver transplantation to those responding to chemotherapy [15]. Several recent studies confirmed the beneficial effect of adjuvant cytostatic therapy after transplantation for hepatocellular carcinoma [6, 11, 13]. However, these studies only comprised a

small number of patients; in the Pittsburgh Study, 11 vs 14 patients were compared. The studies referred to have therefore been criticized repeatedly, and adjuvant chemotherapy is consequently not a commonly accepted procedure in most liver transplant centers. A prospective randomized study comprising a large number of patients and aiming at confirming or excluding the benefit of adjuvant cytostatic therapy is still lacking.

However, it has not been explored thoroughly whether chemotherapy interferes with graft rejection. Beyond that, a precise understanding of the interactions between tumor growth and graft rejection is necessary, especially with regard to immunosuppressive (and adjuvant cytostatic) therapy. In the literature, we could neither find studies that investigate these complex interactions nor a generally applicable and suitable experimental model.

In our study, we introduced a new model that combines a transplantation and tumor model in rats. For the transplantation model, we performed heterotopic heart grafting with the Lewis-Brown Norway rat as donor and Lewis rat as recipient [1]. Heart grafting was used instead of allogeneic liver transplantation, on the one hand because it is easy to carry out, and on the other hand since the cessation of heart beat is a precise indicator of graft rejection. Switching over to heart transplantation seemed admissible since we were interested in the immunological problem of graft rejection and not in the function of the organ itself. The Novikoff hepatoma served as tumor model because its morphology and biological behavior are very close to that of hepatocellular carcinoma [8, 10].

In a first step, we tested the influence of the cytostatic drug on tumor growth. According to clinical experience that proved epirubicin to be very effective against hepatocellular carcinoma [12], a marked tumor regression could be seen. Furthermore, epirubicin was able to diminish the tumor volume-increasing effect of cyclosporine. Our experimental results support the clinical experience that adjuvant chemotherapy is beneficial after transplantation for malignancy.

In a second step, we examined the influence of epirubicin on graft rejection. Graft survival was prolonged significantly and augmented the immunosuppressive effect of cyclosporine.

The immunomodulating impact of cytostatic drugs, demonstrated in our study by the extension of graft survival, is a very interesting phenomenon. The immunohistological examination of the tumors was able to shed light on the reason for this phenomenon: it revealed a markedly reduced expression of W 3–25, thus indicating suppression of T CD4 cells. On the other hand, the immunosuppression evoked by the cytostatic drug might either have been provoked by the high dosage (6 mg/ kg) used in our experimental design, or could be specific to epirubicin: using mitomycin C or 5-fluorouracil in the same model did not prolong graft survival [16].

The potential immunomodulating effect of an adjuvant cytostatic regimen is an almost accepted phenomenon. In the precycolosporine-era, antimetabolic drugs were used as immunosuppressants since 6-mercaptopurine or cyclophosphamide were applied to transplantation. However, up to the present time, no precise data exist concerning this problem because neither experimental nor clinical studies have so far been performed.

An additional supplementary aim of our study was to investigate the interactions between tumor growth and graft rejection. In a previous study, we could show that graft survival is prolonged in the presence of a malignant tumor [16]. In this study, we demonstrate that neither does a solid tumor resecceted before transplantation have an influence on graft survival, nor does an allogeneic graft have an influence on tumor growth. As a consequence, the above-mentioned reactions were not influenced by interactions between the tumor and the allogeneic graft, but only by the administered drugs.

The attempt to imitate the clinical situation, i. e., liver transplantation for liver cancer, by an experimental design which combines a heart transplantation and tumor model obviously has its limitations. In contrast to our model, for example, the primary tumor is removed in a clinical situation and potential interactions must aim at subclinical micrometastases. Furthermore, our model only describes the effect of the cytostatic drug on tumor growth and graft rejection, without providing an answer to the immunological processes of these phenomenon.

In summary, however, our results demonstrate despite these limitations that an adjuvant chemotherapy after transplantation does not interfere negatively with graft survival. Our experimental data therefore support clinical studies that adjuvant cytostatic treatment might be a valuable tool in reducing the high rate of tumor recurrence.

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