Transpl Int (2005) 17: 687–691
DOI 10.1007/s00147-004-0752-1

ORIGINAL ARTICLE

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Gemcitabine does not prevent acute rejection of the transplanted liver in rats

Received: 21 July 2003
Revised: 12 February 2004
Accepted: 2 July 2004
Published online: 3 December 2004

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Abstract Our study was designed to determine effect of gemcitabine on acute rejection of liver in rats. Liver transplantation was performed in rats of the Dark Agouti (DA) and Lewis (LEW) strains. Recipients were divided into three groups: A, DA-to-LEW without immunosuppression; B, DA-to-LEW, treated with cyclosporine A; C, DA-to-LEW, treated with gemcitabine. Immunosuppressants were subcutaneously injected for seven consecutive days after transplantation. On day 7, blood samples and liver graft tissue specimens were harvested. Group A showed severe rejection changes (RAI 8/9); in group B no rejection changes were present (RAI 0/9), and in group C moderate rejection changes were observed (RAI 6/9). Differences were significant between B vs C and A vs C groups; $P < 0.05$. Serum creatinine and urea levels in the gemcitabine group were significantly lower than those in the cyclosporine A group. We did not confirm gemcitabine ability to prevent liver allograft rejection.

Keywords Liver transplantation · Acute rejection · Immunosuppression · Gemcitabine · Rat

Introduction

Gemcitabine (2'2'-difluorodeoxycytidine, dFdC) is a pyrimidine anti-metabolite, originally developed as an antiviral agent. However, given its cytotoxicity, it was later approved for adjuvant therapy for a broad range of solid tumours [1]. Several trials are currently ongoing and are designed to assess the radio-sensitization of various animal and human tumour cells allowing gemcitabine administration [2]. Recently, gemcitabine has been used as an immunosuppressive agent in an experimental model [3, 4].

On entering cells, gemcitabine is phosphorylated progressively, by deoxycytidine kinase (dCK), to diphosphate and triphosphate, which is the main control step in dFdC activation; the result is a triphosphate. The incorporation of the dFdC triphosphate in the DNA chain is presumably the main mechanism of gemcitabine's anti-neoplastic activity, as it inhibits DNA synthesis thus causing cell death [5].

For dFdC to be able to damage the cell, it must be phosphorylated by deoxycytidine kinase. Given the high levels of this enzyme in lymphocytes, it is particularly these cells that are damaged. This also explains

gemcitabine's lymphotoxicity as its most serious side effect.

The effect of gemcitabine on liver graft rejection has not been assessed to date. As a result, our study was designed to determine the ability of gemcitabine to prevent acute liver rejection in the rat.

Material and methods

Animals

A model of acute liver rejection between fully allogenic strains was employed. Adult inbred male rats of the strains Dark Agouti (DA, RT.1^{av1}) and Lewis (LEW, RT.1^{l}) weighing 260 g to 340 g were obtained from Harlan, the Netherlands (DA) and from Charles River, Germany (LEW). The rats were housed in conventional cages with free access to rodent diet and water. All animals received care according to the national guidelines for animal care, and the project was approved by the ethics committee of the regional authorities in compliance with Czech law (Act 246/1992).

Groups

Animals were divided into three groups: A ($n=6$) DA-to-LEW, without an immunosuppressive; B ($n=5$) DA-to-LEW treated with cyclosporine A (CyA; Sandimmun Neoral, Novartis Pharma AG, Switzerland) 2.5 mg/kg per day; C ($n=5$) DA-to-LEW, treated with gemcitabine (Gemzar, Lilly France S.A., France) 100 μg/kg per day. Immunosuppression was subcutaneously administered once daily, starting with transplantation day. All animals were killed on post-transplant day 7, when blood samples and liver graft specimens were obtained.

Orthotopic liver transplantation

Orthotopic liver transplantation was performed by a modified method [6], without revascularization of the hepatic artery, as originally described by Kamada, Clane and Lee [7, 8], under isoflurane inhalation anaesthesia (Forane, Abbot Laboratories, UK). In brief, the donor liver was flushed through the portal vein with ice-cold sterile saline solution. We initiated the liver grafting by anastomosing the suprahepatic vena cava with 8-0 running sutures. The anastomoses of the portal vein and infrahepatic vena cava were performed with running 9-0 nylon suture, and the bile duct continuity was restored by 22G stent anastomosis. The death of an animal within 7 days was attributed to a technical error, and the animals were not included to the study.

Histology

Liver graft specimens were fixed in 10% neutral formalin solution and processed according to the routine protocol. The 5-μm cut sections were stained with haematoxylin and eosin. Histopathological features were assessed semiquantitatively by the Banff schema for grading of acute liver allograft rejection [9].

Biochemistry

Biochemical parameters, i.e. bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), gamma glutamyl transpeptidase (GGT), albumin, creatinine and urea were assessed on post-transplant day 7.

Statistical analysis

The Mann–Whitney U-test (acute rejection score) and the Kruskal–Wallis univariant analysis (biochemical values) were used for statistical analysis of data. P values below 0.05 were considered significant.

Results

Histological findings in liver allograft tissue specimens were different in each group, and the differences between A vs B, B vs C, and A vs C were statistically significant ($P<0.05$). Morphological changes consistent with severe acute rejection were found in all specimens of group A (untreated animals) RAI 8/9 (Fig. 1). Group B (CyA-treated) showed no evidence of rejection (RAI 0/9) (Fig. 2), whereas there were histological features of moderate acute rejection in the gemcitabine-treated group (RAI 6/9). Serum creatinine and urea levels were lower ($P<0.05$) in all gemcitabine-treated animals than in CyA-treated animals. Bilirubin levels were likewise significantly lower in groups B and C than in group A ($P<0.05$); however, no significant differences were detected in ALT, AST, GGT, and AP (Fig. 2).

Discussion

The main side effects of immunosuppressives used as standard agents in liver transplantation (calcineurin inhibitors, cyclosporine, and FK 506) include nephrotoxicity, diabetogenic effects and neurotoxicity. Mycophenolate mofetil may cause gastric complaints and leukopenia [8, 9]. Other cytotoxic immunosuppressants—methotrexate, cyclophosphamide and azathioprine—which belong, with gemcitabine, in the

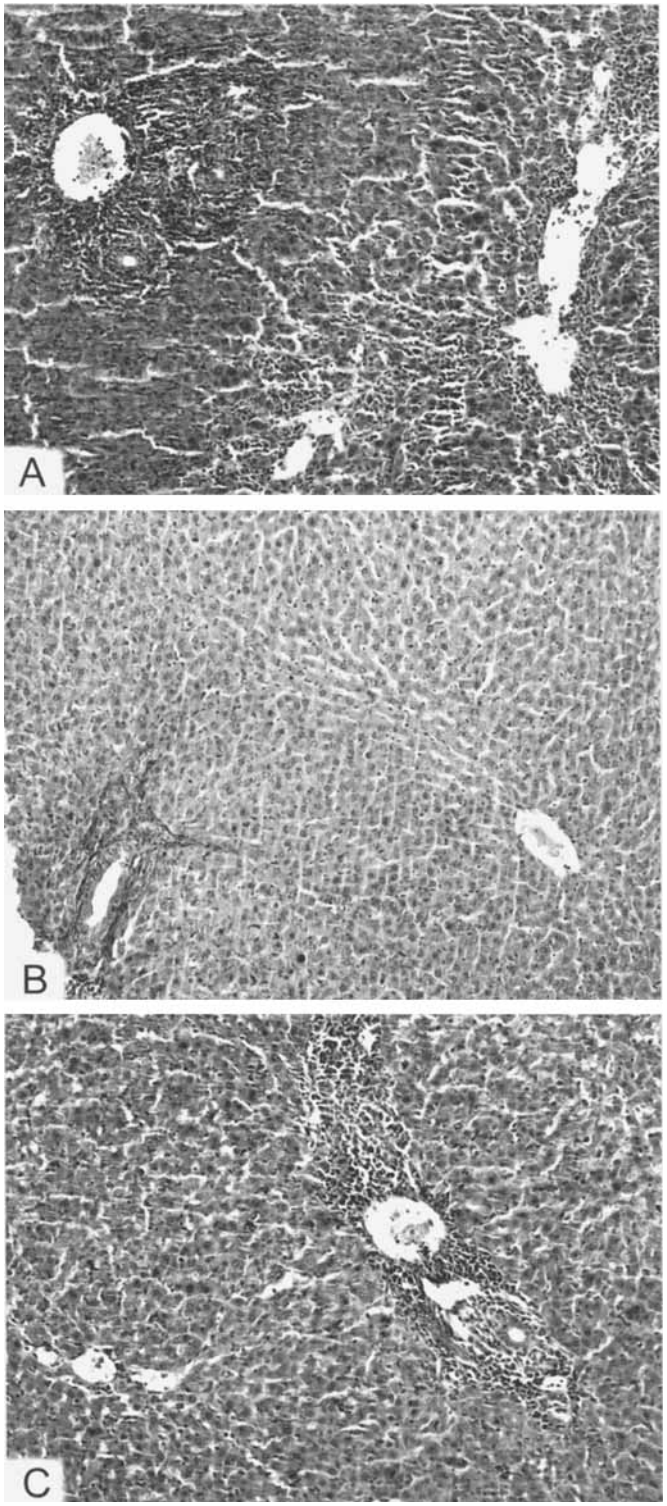

Fig. 1 Histological findings. **A** Severe acute liver allograft rejection. Portal tract contains a dense mixed inflammatory infiltrate. There are morphological features of bile-duct damage. Portal and hepatic venules show subendothelial inflammation with perivenular inflammation that extends into the surrounding parenchyma and is associated with perivenular hepatocyte necroses. **B** Liver parenchyma without any rejection changes and inflammation. **C** Moderate acute liver allograft rejection. Portal tract shows changes similar to those seen in **A**. There are no centrilobular necroses associated with perivenular inflammation

anti-metabolite group of drugs, inhibit many types of proliferating cells. This is the reason for the wide spectrum of side effects, and, due to their low specificity to suppress, firstly, lymphocyte formation, they have been abandoned as a main immunosuppressive medication since the production of cyclosporine.

Another agent, sirolimus, is used with caution, as its use has been associated with the formation of hepatic artery thromboses [10]. It would be most helpful if the range of immunosuppressive agents were to be expanded, by the addition of drugs with fewer side effects that could be potentially used in liver transplantation. One of these agents could be gemcitabine, a drug showing, at standard cytotoxic dose, relatively low total toxicity. Its immunosuppressive action was first assessed by a team headed by Margreiter [3] in a model of heart transplantation in the rat, with encouraging preliminary results. They confirmed the hypothesis that multiple administration of low-dose dFdC has a primary effect on immunocompetent cells. The authors tested, in vitro, the effects of gemcitabine on the inhibition of T-lymphocyte colony formation after T-lymphocyte stimulation. Peripheral blood mononuclear cells (PBMCs) were cultured with phytohaemagglutinin (PHA) and with various dFdC levels in a micro-agar system. It was confirmed that the PHA-induced lymphocyte proliferation is inhibited by dFdC in a dose-dependent manner, with 50% inhibition at a concentration of 3.25 ± 0.9 nmol/l. They tested in vivo dFdC in heart allograft transplantation in the rat. When using increasing gemcitabine doses, they noted that graft survival was extended with rising doses, starting with a dose of 75 μg/kg per day. Treatment with a dose of 100–125 μg/kg per day was capable of extending graft survival times from an approximate 8 days to 150 days. While lower doses were not effective, higher doses were toxic and decreased overall survival times, although the graft remained functional. The most common cause of death was lung infection. A positive effect of dFdC was, later, also confirmed by Jung et al. [4]. Those authors tested dFdC in rat models of acute kidney and heart rejection and in a model of accelerated acute heart rejection. Accelerated rejection was induced by previous *Lewis* recipient sensitization with skin from Brown Norway donors 7 days prior to heart transplantation. In the first model of heart transplantation, rejection was delayed from day 8 to day 37 (130 μg/kg per day) or, alternatively, from day 8 to day 69 in renal transplantation (150 μg/kg per day), whereas the delay was from

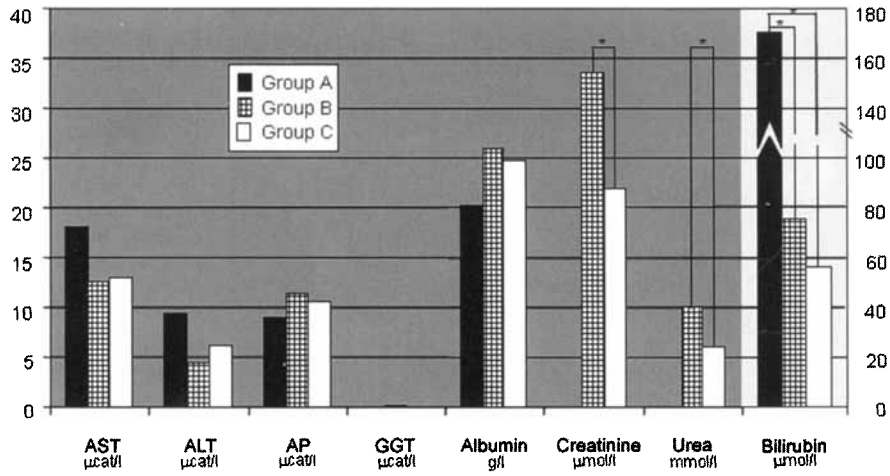

Fig. 2 Peripheral blood levels of selected liver and renal function parameters. Group A: animals after liver transplantation without further therapy. Group B: animals treated with cyclosporine A. Group C: animals treated with gemcitabine

24–36 h to 5 days (150 µg/kg per day). This outcome is at variance with that reported by Margreiter et al., who described serious side effects with such high doses, such as excess immunosuppression with irreversible myelotoxicity and death of animals with functioning graft due to infection. Unfortunately, histological findings from kidney, liver and heart grafts were not presented [4].

Unlike the above trials that monitored graft survival times, our study was designed to determine histological findings in liver graft specimens. We used a time-tested model of acute liver graft rejection between fully allogenic DA and LEW strains [11]. The immunosuppressive effect was assessed on the basis of histological features, which correlated closely with post-transplantation survival times [12]. The Banff schema for the grading of liver allograft rejection was used. As we wanted to use, as the endpoint, results from histology, it was critical for us to obtain specimens from grafts before the animals had died from allograft failure. Cyclosporin A at 2.5 mg/kg per day and gemcitabine at 100 µg/kg per day had previously been confirmed as effective by other authors [3, 6].

Our study, originally seeking to determine whether or not gemcitabine would affect the development of acute rejection in experimental liver allograft, did not confirm previous data suggesting dFdC as a powerful immunosuppressive. Histology suggested gemcitabine was unable to prevent acute hepatic rejection. Nevertheless, the dose–response experiment in the liver transplant model, comparing histological assessment with survival of animals, should be done to confirm this. In theory, a higher dose could be used; this, however, at the expense of increased side effects.

Biochemical results demonstrated significant differences in creatinine and urea levels between groups B and C, suggesting lower nephrotoxicity of dFdC than of CyA. A significant difference was also found in bilirubin levels. By contrast, the differences in AST, ALT, GGM, and AP were statistically non-significant, which may have been due to the small number of animals.

In conclusion, our results in an experimental model have shown that gemcitabine cannot be used as an immunosuppressive agent in monotherapy after liver transplantation. It would be appropriate to test its use in combination with other agents in future trials.

Acknowledgements This study was supported by VZ/CEZ:L17/98:00023001.

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