Transpl Int (2001) 14: 153–158

ORIGINAL ARTICLE

Yu-Chun Lin
Shigeru Goto
Tair-Long Pan
Yi-Ren Hong
Chen-Lung Lin
Roger Lord
Kuei-Chen Chiang
Chia-Yun Lai
Hui-Peng Tseng
Li-Wen Hsu
Sachio Iwashita
Seigo Kitano
Chao-Long Chen

Identification of two down-regulated genes in rat liver allografts by mRNA differential display

Received: 14 December 1999
Revised: 28 September 2000
Accepted: 16 March 2001

Y. Lin · S. Goto (✉) · T. Pan · C. Lin
K. Chiang · C. Lai · H. Tseng · L. Hsu
C. Chen
Department of Surgery,
Chang Gung Memorial Hospital,
123 Ta-Pei Rd, Niao-Sung,
83301 Kaohsiung, Taiwan
e-mail: shigeru@adm.cgmh.org.tw
Tel.: + 886-7-7317123/ext. 8097
Fax: + 886-7-7324855

Y. Hong
Department of Biochemistry,
Kaohsiung Medical University,
80708 Kaohsiung, Taiwan

R. Lord
Discipline of Surgery,
University of Tasmania, GPO Box 252–28,
Hobart, Tasmania 7001, Australia

S. Iwashita · S. Kitano
Department of Surgery I,
Oita Medical University, 875 Oita, Japan

Abstract Total RNA differential display (DD) using random primers was performed for rat orthotopic liver transplantation (OLT) models. DA (RT1^a) donor livers were transplanted into DA, PVG (RT1^c), and LEW (RT1^l) recipients: (1) syngeneic OLT (DA-DA): no rejection occurs; (2) allogeneic OLT (DA-PVG): rejection occurs, but is naturally overcome without immunosuppression; (3) allogeneic OLT (DA-LEW): animals die of acute rejection within 14 days. cDNA was isolated from selected bands, re-amplified for sequencing, and confirmed by Northern blots. Two down-regulated genes were observed in day-7 allogeneic OLT livers (DA-PVG, DA-LEW), while they were consistently expressed in day-7 syngeneic OLT (DA-DA) livers. These two genes were identified as α-glutathione sulfotransferase (α-GST) Ya gene and estrogen sulfotransferase (EST), respectively. Northern blots confirmed that their expression was down-regulated in OLT (DA-PVG) livers on days 7–26 and gradually restored. The mRNA expression of GST and EST may be good markers to predict rejection or induction of tolerance.

Keywords Rat · Liver transplantation · Tolerance · Glutathione sulfotransferase · Estrogen sulfotransferase

Abbreviations *DD* Differential display · *EST* Estrogen sulfotransferase · *GST* Glutathione sulfotransferase · *OLT* Orthotopic liver transplantation · *PCR* Polymerase chain reaction

Introduction

In certain class I and class II disparate rat strain combinations such as DA (RT1^a) donor and PVG (RT1^c) recipient, orthotopic liver transplantation (OLT) results in a state of long-lasting and donor-specific tolerance without pharmacological immunosuppression, whereas skin, heart, and renal allografts undergo acute rejection [10]. Although an early rejection reaction develops over the first 2 weeks after OLT (DA-PVG), this response resolves to give tolerance to donor-type skin grafts by 60 days [8]. On the other hand, when the same DA donor livers are transplanted into LEW recipients, all recipients die of acute rejection within 14 days. Therefore, the fate of liver grafts in the rat is genetically determined and dependent on the particular combination of donor and recipient strains.

Although conventional measurements of mRNA transcript levels confined to rejection-related genes have been performed substantially, little is known about specific molecular factors involved in the pathogenesis of acute liver rejection [5, 15]. Differential display

(DD) of mRNA, which permits the simultaneous identification of up-regulated genes as well as down-regulated genes, has been used to identify unknown mediators or known mediators as previously implicated in rat chronic cardiac rejection [18]. However, as far as we know, this technique has not been applied to liver allograft rejection. The molecular identification and characterization of rejection-regulated genes will provide important insights not only into fundamental mechanisms of rejection, but also into the application of gene therapy against rejection [6, 14]. In the present study, we adapted the DD technique for the evaluation of gene expression patterns directly for transplanted livers by comparing mRNA profiles in OLT (DA-PVG or DA-LEW) livers during acute rejection with those in control livers subjected to syngeneic transplantation.

Materials and methods

OLT and liver samples

Male DA ($RT1^a$), PVG ($RT1^c$), and LEW ($RT1^l$) rats weighing 250–300 g were obtained from Harlan UK Limited, Oxford, United Kingdom. All animals were maintained in specific pathogen-free animal facilities with water and commercial rat food provided ad libitum. OLT was performed according to the method described by Kamada and Calne [9]. DA livers were orthotopically transplanted into DA ($n = 18$), PVG ($n = 18$), and LEW ($n = 18$) recipients. As reported previously [11], OLT (DA-PVG) rats naturally overcome rejection while all OLT (DA-LEW) rats die from acute rejection within 14 days. Syngeneic OLT (DA-DA) rats were used as controls since no rejection occurs and rejection-related factors are eliminated. One OLT (DA-PVG) rat and one OLT (DA-LEW) rat of a total of 54 OLT rats died due to technical failure within 24 h after OLT and were excluded from the experimental groups. Three animals were killed at each time point (postoperative days 2, 7, 14, 26, 40, and 67) after OLT and the livers used for RNA isolation and differential mRNA display.

RNA isolation and mRNA DD

Rat livers were harvested at various time points after transplantation and quick-frozen in liquid nitrogen. Total RNA was extracted from liver tissue using TRI-Reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. mRNA DD was performed using a Delta Differential Display Kit (Clontech Laboratories, Palo Alto, Calif.). After DNase I treatment, total RNA (2 μg) was reverse-transcribed with 200 U of Moloney murine leukemia virus reverse transcriptase (Clontech Laboratories) in the presence of 1 pmol oligo-d(T) anchor primer. The cDNA was then amplified by polymerase chain reaction (PCR). The PCR reaction mixture (20 μl) included arbitrary primer sets (Clontech Laboratories), dNTP, α-p^{33}dATP (Amersham Pharmacia Biotech, Buckinghamshire, UK), and Taq enzyme (Roche Molecular Biochemicals, Mannheim, Germany). The cycling conditions were 5 min at 94 °C, 5 min at 40 °C, 5 min at 72 °C (1 cycle), 2 min at 94 °C, 5 min at 40 °C, 5 min at 72 °C (2 cycles), 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C (30 cycles). The radioactive PCR amplification products were analyzed by electrophoresis in denaturing 6 % polyacrylamide gels. Differentially expressed cDNA bands were recovered from sequencing gels and re-amplified with the same primer sets in high-stringency conditions without radioisotope. The re-amplified PCR products were used as templates for random priming and for cloning into pGEM-T Easy Vector (Promega, Madison, Wis.).

Northern blot analysis

Samples of total RNA (30 μg) were fractionated in 0.1 M sodium phosphate/1 % agarose gels and transferred onto Zeta-Probe membrane (Bio-Rad Laboratories, Hercules, Calif.) by standard capillary blotting techniques. Specific probes were generated by labelling re-amplified or cloned cDNA fragments with digoxigenin-11-dUTP and a Random Primer DNA Labelling Kit (Roche Molecular Biochemicals). Filters were pre-hybridized for 2 h at 50 °C and then hybridized by addition of denaturing probes. After overnight hybridization, blotting filters were washed to a final stringency of 0.1 × SSC-0.1 % SDS at room temperature and then detected by a colorimetric method with NBT/BCIP (Roche Molecular Biochemicals).

TA cloning and DNA sequence analysis

Re-amplified cDNA fragments that produced a specific hybridization pattern on Northern blots, which was the same as the pattern in DD, were cloned into the plasmid pGEM-T Easy Vector using the TA cloning kit (Promega). To obtain the maximum amount of information, we selected at least ten bacterial colonies from one clone for DNA sequencing via the T_7 Sequenase Version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech). The nucleotide sequences obtained were then compared with known sequences by searching the Genbank/EMBL databases by means of the BLAST program.

Results

Differential mRNA display

To identify genes up-regulated or down-regulated during acute rejection, we compared differential mRNA display patterns for day-7 livers from syngeneic OLT (DA-DA) with those for day-7 livers from allogeneic OLT (DA-PVG or DA-LEW) (Fig. 1). Histological examination of the DA liver grafts in either PVG or LEW recipients showed severe acute rejection on day 7, with portal and lobular inflammation, biliary epithelial injury, and endothelialitis (data not shown). We performed PCR amplifications with 15 primer combinations on all five samples in duplicate and identified 13 PCR products, designated bands 1 to 13, that were differentially expressed among naïve, syngeneic, and allogeneic liver tissue. As indicated, bands 9, 10, and 12 are three representative PCR products that were reproducibly present in the naïve livers (lanes 1 and 2) and syngeneic samples (lane 3), but not in the allogeneic samples (lanes 4 and 5) (Fig. 1).

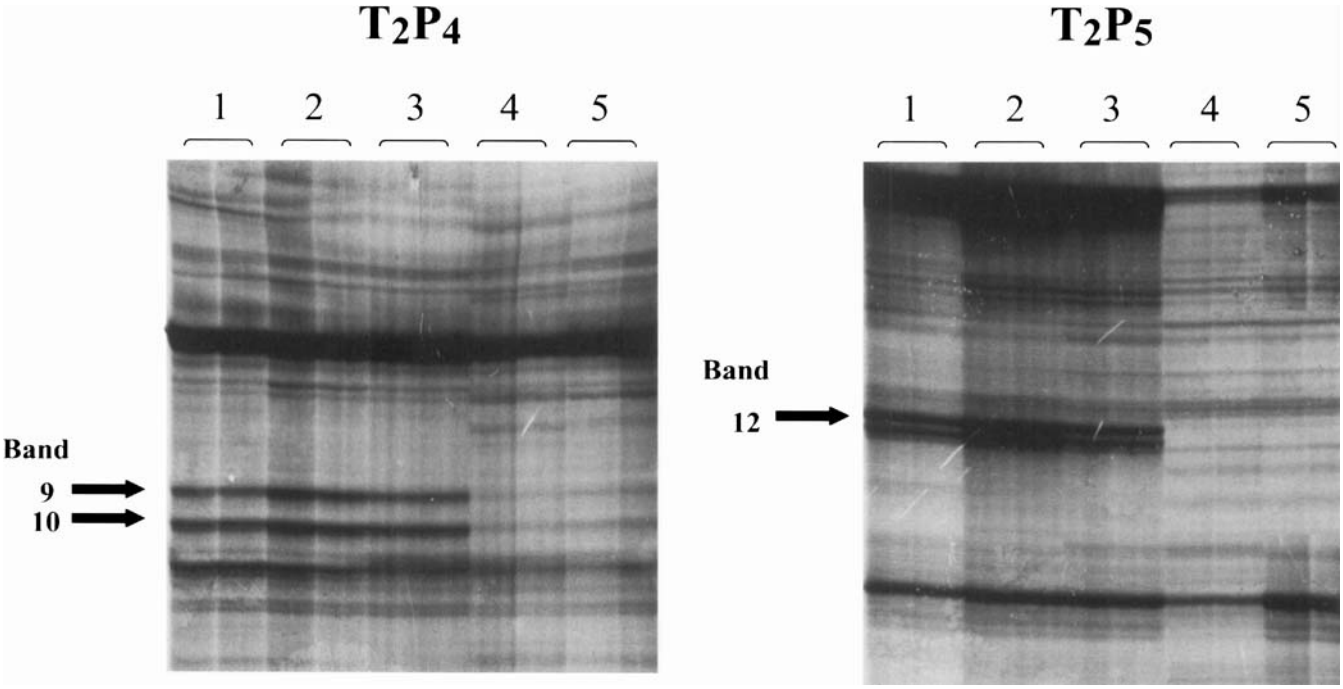

Fig. 1 Differential display comparing RNA from three groups of livers. The first group contains naïve DA livers (*lane 1*) and naïve PVG livers (*lane 2*); the second group, 7-day syngeneic DA livers (*lane 3*); and the third group, 7-day allogeneic DA livers from LEW rats (*lane 4*) and PVG rats (*lane 5*). Liver samples were taken from three individual naïve rats or OLT rats. The results are representative of three separate experiments. Autoradiograms are shown for two primer combinations, T_2P_4 and T_2P_5, which identified three distinct fragments (*arrows*) up-regulated in naïve and syngeneic groups. The anchor primer is T_2 (5′CATTATGCTGAGTGATATCTTTTTTTTTAC-3′), the arbitrary primers are P_4 (5′ATTAACCCTCACTAAATGCTGGTAG-3′) and P_5 (5′ATTAACCCTCACTAAAGATCTGACTG-3′)

RNA (Northern) blot analysis and sequence homology of two down-regulated genes

To confirm the gene expression patterns observed in the DD study, we recovered the 13 selected bands from the polyacrylamide gels and re-amplified them. The re-amplified PCR fragments were used as probes for RNA blot analysis prepared with RNAs from naïve, syngeneic, and allogeneic livers. When used as probes, three of the 13 PCR-amplified fragments (bands 9, 10, and 12) generated specific hybridization patterns that were also observed in the DD (Fig. 2). Three of the 13 re-amplified PCR fragments hybridized nonspecifically to all five samples and were not studied further. The remaining seven probes did not detect any RNA transcripts. However, it is possible that the levels of these RNA transcripts were below the sensitivity of the RNA blot analysis.

The PCR products that generated specific hybridization patterns were then cloned. To get as much possible information from the bacterial colonies, we selected at least ten colonies from one clone for DNA sequencing. Unexpectedly, the cloned cDNA fragments from bands 9 and 10 were found to be 100% homologous to rat estrogen sulfotransferase (EST). The other cDNA fragment (510 bp) from band 12 was 96% identical to rat glutathione sulfotransferase (GST) Ya gene.

Time course

We also used the cloned fragments in RNA blot analyses to evaluate changes in gene transcript levels over time (Fig. 3). In the tolerogeneic OLT (DA-PVG) model, allografted livers undergo acute rejection as early as day 3, when lymphocyte infiltration begins. At day 7, infiltration is more evident and is overcome as late as day 26, when only mild portal infiltration by plasma cells is seen. For two cDNA clones, strong signals were detected in naïve DA livers prior to OLT. Hybridization was visible at day 2, became faint and not detectable at days 7 and 14, and then was gradually restored by days 40 and 67.

Discussion

Liver transplantation has become a highly efficient treatment for patients with end-stage liver cirrhosis and fulminant liver failure. However, despite improvements

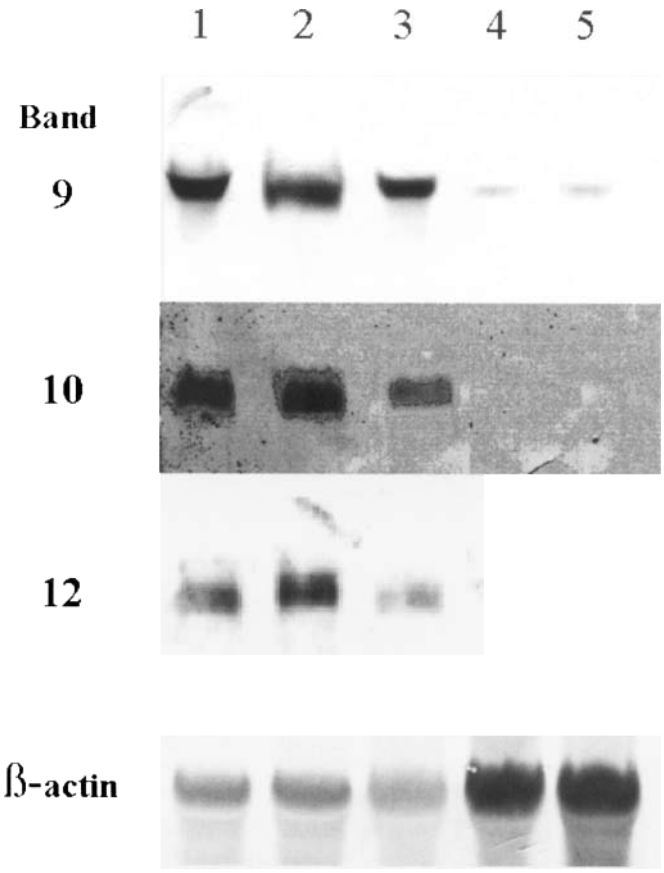

Fig. 2 RNA blot analysis confirming specific gene expression for differential display bands 9, 10, and 12. Total RNA was obtained from DA and PVG naïve livers (*lanes 1 and 2*), day-7 syngeneic DA liver (*lane 3*), and day-7 allogeneic DA livers from a LEW rat (*lane 4*) and a PVG rat (*lane 5*) and hybridized with cDNA probes generated by PCR re-amplification of bands recovered from differential display gels. RNA loading was evaluated by probing the same blot with rat liver β-actin

in immunosuppression, 50–70% of liver transplant patients experience one or more acute rejection episodes [19]. There are, however, no reliable and specific markers for a diagnosis of acute rejection.

α-GST located on hepatocytes is a cytosolic enzyme of the liver with a short half-life. It is still controversial whether serum levels of α-GST can be used as a specific marker for acute cellular rejection in liver transplantation [7, 12, 17]. The serum level of α-GST is at least in part a sensitive marker for hepatocellular integrity or acute liver injury in liver donors and recipients [12]. However, little is known about the gene expression of α-GST in OLT models. In our rat OLT models, hepatocytes were damaged by the rejection reaction, and α-GST may be released to serum from hepatocytes destroyed during the rejection reaction. On the other hand, the destruction of hepatocytes may decrease the expression of α-GST mRNA in liver allografts during the rejection phase following OLT, as shown in our results. Additionally, the restoration of α-GST mRNA expression in OLT (DA-PVG) livers matched the timing of establishment of tolerance when the rejection reaction was naturally overcome and hepatocytes were no longer being damaged.

Mammalian EST sulfurylates the hydroxyl group of estrogenic steroids by transferring the sulfate from a co-substrate adenosine 3′phosphate-5′phosphosulfate. Sulfurylated steroids do not bind to the estrogen receptor with high affinity and, therefore, are hormonally inactive [4]. Male rats have EST in the liver to inactivate estrogens [19]. In the present study using young male rats (10–12 weeks), expression of EST mRNA was strong in livers of naïve rats prior to OLT. Interestingly, however, hepatic EST mRNA was down-regulated during the rejection reaction in allogeneic OLT (DA-PVG or DA-LEW), while this gene is intact in livers of syngeneic OLT (DA-DA). Since no immunosuppressive drugs were given in these allogeneic OLT models, the recipients may require estrogen in the allografts by down-regulating EST in order to combat the rejection reaction.

Although the precise role of estrogen in rejection is unclear, the immunological function of estrogen has been reported to regulate extrathymic T cells in the liver [13] or intestine [3]. It has been reported that estrogen administration activates an extrathymic pathway of T-cell differentiation in the liver and reciprocally inactivates the intrathymic pathway [13]. Further, it is well known that the liver is a hostile environment for activated CD8 cells, and this might cause peripheral T-cell deletion, which may account for the unique features of liver allograft tolerance. However, it is still unclear why activated CD8 T cells first accumulate in the liver and, then, how these CD8-positive cells receive an apoptotic signal at that site. Estrogen is likely to play an important role in the liver allograft by inducing depletion of donor-reactive CD8 T cells derived from the thymus.

In terms of intragraft cytokine mRNA expression, IL-2 mRNA was detected specifically in rejection biopsies concurrent with graft dysfunction [2]. Intragraft IL-4 or IL-15 mRNA expression correlated significantly with spontaneously resolving rejections [1, 2]. However, others have reported that there is no major difference in the extent of up-regulation of these cytokine mRNAs between tolerance and rejection [16]. Thus, it is still unclear whether intragraft mRNA expression of certain cytokines can predict rejection or tolerance. Intragraft cytokine mRNA profiles may be different among various organ allografts and also between the states of rejection and tolerance treated with or without immunosuppressive drugs. In other similar reports using DD assays, the differentiated genes studied have always been focused on up-regulated genes specific to the

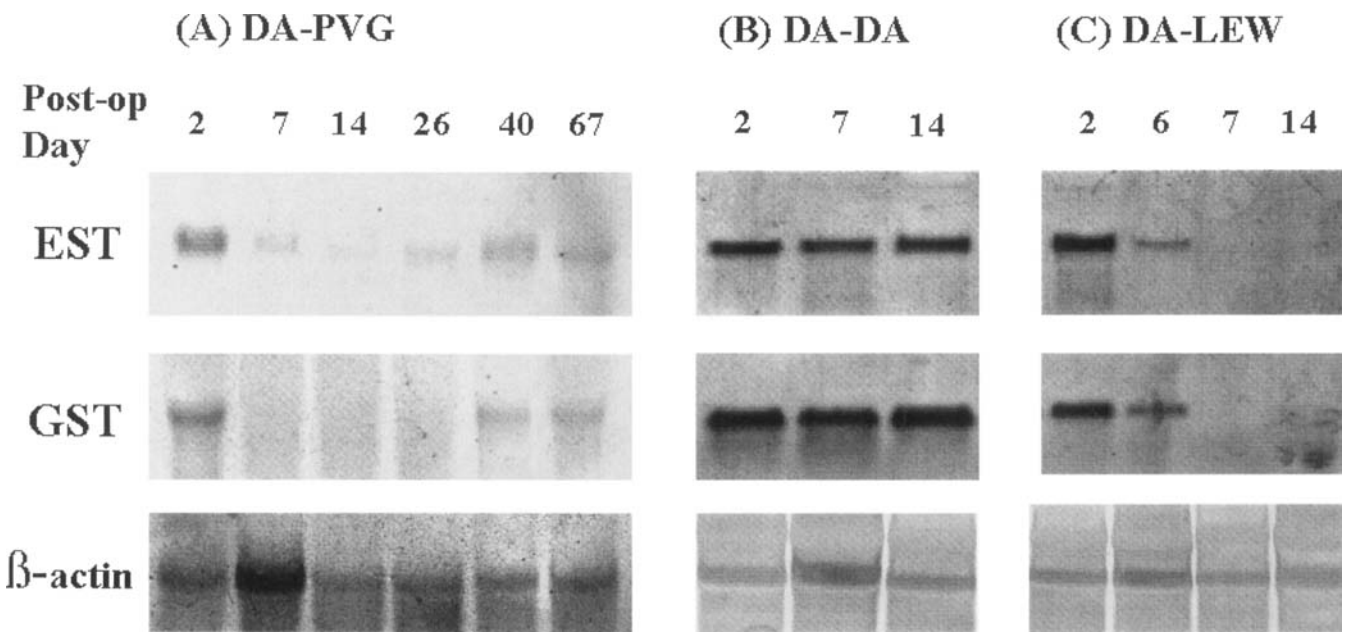

Fig. 3 RNA blot analysis of (**A**) allogeneic DA rat livers from PVG rats at days 2, 7, 14, 26, 40, and 67, (**B**) syngeneic DA rat livers from DA rats at day 2, 7, and 14, and (**C**) allogeneic DA rat livers from LEW rats at days 2, 6, 7, and 14, with two cloned cDNA fragments, estrogen sulfotransferase (*EST*) and glutathione sulfotransferase (*GST*)

experimental groups. Our results clearly demonstrated that the identification of down-regulated genes by DD is also informative. In OLT (DA-PVG), the rejection reaction is serious on days 7–14 and naturally overcome without any immunosuppressive drugs, followed by donor-specific tolerance. The down-regulated expression of GST and EST mRNA has been gradually restored in accordance with termination of rejection and subsequent establishment of tolerance. Although further studies including the effect of immunosuppressive drugs on intragraft GST and EST mRNA expression are required, our results suggest that EST and GST recovery may be a good marker of tolerance by which we will be able to determine when immunosuppressive therapy can safely be ceased for long-term survival transplant patients.

Acknowledgements This work was supported by grants from the National Science Council (NSC-89–2314-B-182–055) and National Health Research Institute (NHRI-GT-EX89S904L).

References

1. Baan CC, Metselaar HJ, Mol WM, Tilanus HW, IJzermans JM, Zondervan PE, Schalm SW, Niesters HG, Weimar W (1996) Intragraft IL-4 mRNA expression is associated with down-regulation of liver graft rejection. Clin Transplant 10: 542–549
2. Baan CC, Niesters HG, Metselaar HJ, Mol WM, Loonen EH, Zondervan PE, Tilanus HW, IJzermans JM, Schalm SW, Weimar W (1998) Increased intragraft IL-15 mRNA expression after liver transplantation. Clin Transplant 12: 212–218
3. Boll G, Reimann J (1996) Oestrogen treatment depletes extrathymic T cells from intestinal lymphoid tissues. Scand J Immunol 43: 345–350
4. Demyan WF, Song CS, Kim DS, Her S, Gallwitz W, Rao TR, Slomczynska M, Chatterjee B, Roy AK (1992) Estrogen sulfo-transferase of the rat liver: complementary DNA cloning and age- and sex-specific regulation of messenger RNA. Mol Endocrinol 6: 589–597
5. Egawa H, Martinez OM, Quinn MB, Villanueva JC, So S, Esquivel CO, Krams SM (1995) Acute liver allograft rejection in the rat. An analysis of the immune response. Transplantation 59: 97–102
6. Geissler EK, Graeb C, Tange S, Guba M, Jauch KW, Scherer MN (2000) Effective use of donor MHC class I gene therapy in organ transplantation: prevention of antibody-mediated hyperacute heart allograft rejection in highly sensitized rat recipients. Hum Gene Ther 11: 459–469
7. Hughes VF, Trull AK, Joshi O, Alexander GJ (1998) Monitoring eosinophil activation and liver function after liver transplantation. Transplantation 65: 1334–1339
8. Kamada N (1985) The immunology of experimental liver transplantation in the rat. Immunology 55: 369–389

9. Kamada N, Calne RY (1983) A surgical experience with five hundred thirty liver transplants in the rat. Surgery 93: 64–69
10. Kamada N, Davies HS, Roser B (1981) Reversal of transplantation immunity by liver grafting. Nature 292: 840–842
11. Lord R, Goto S, Vari F, Edwards-Smith C, Chiba S, Sunagawa M, Lynch SV (1997) Differences in the rate of donor leucocyte migration between natural and drug-assisted tolerance following rat liver transplantation. Clin Exp Immunol 108: 358–365
12. Nagral A, Butler P, Sabin CA, Rolles K, Burroughs AK (1998) α-glutathione-S-transferase in acute rejection of liver transplant recipients. Transplantation 65: 401–405
13. Okuyama R, Abo T, Seki S, Ohteki T, Sugiura K, Kusumi A, Kumagai K (1992) Estrogen administration activates extrathymic T cell differentiation in the liver. J Exp Med 175: 661–669
14. Shinozaki K, Yahata H, Tanji H, Sakaguchi T, Ito H, Dohi K (1999) Allograft transduction of IL-10 prolongs survival following orthotopic liver transplantation. Gene Ther 6: 816–822
15. Shintaku S, Ohdan H, Yamamoto H, Miyata Y, Hayashi A, Ito H, Fukuda Y, Asahara T, Dohi K (1998) Expression of bcl-2 homologue mRNAs in rat liver allograft: rejection-induced cell apoptosis is associated with upregulation of bax and bcl-xs expression. Transpl Int 11: S284–S288
16. Sun J, McCaughan GW, Matsumoto Y, Sheil AG, Gallagher ND, Bishop GA (1994) Tolerance to rat liver allografts. I. Differences between tolerance and rejection are more marked in the B cell compared with the T cell or cytokine response. Transplantation 57: 1349–1357
17. Trull AK, Facey SP, Rees GW, Wight DG, Noble-Jamieson G, Joughin C, Friend PJ, Alexander GJ (1994) Serum α-glutathione S-transferase – a sensitive marker of hepatocellular damage associated with acute liver allograft rejection. Transplantation 58: 1345–1351
18. Utans U, Liang P, Wyner LR, Karnovsky MJ, Russell ME (1994) Chronic cardiac rejection: identification of five upregulated genes in transplanted hearts by differential mRNA display. Proc Natl Acad Sci USA 91: 6463–6467
19. Wiesner RH (1996) Is hepatic histology the true gold standard in diagnosing acute hepatic allograft rejection? [editorial; comment]. Liver Transpl Surg 2: 165–167