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# Better-Surviving Liver Grafts by the Injection of Anti-CD2 Antibody: The Important Roles of Host $CD8^+$ and $CD2^+CD28^+$ T Cells in Chronic Graft Rejection and $\beta$ Type Platelet-Derived Growth Factor Receptor (PDGFR-$\beta$) Expression on Apoptotic Liver Grafts

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NAKATSUJI, T. *Better-Surviving Liver Grafts by the Injection of Anti-CD2 Antibody: The Important Roles of Host $CD8^+$ and $CD2^+CD28^+$ T Cells in Chronic Graft Rejection and $\beta$ Type Platelet-Derived Growth Factor Receptor (PDGFR-$\beta$) Expression on Apoptotic Liver Grafts.* Tohoku J. Exp. Med., 1999, **187** (3), 215-225 —— Syngeneic liver grafts were implanted in the livers of 22 LEW/Sea strain rats. To prolong the graft survival, anti-CD2 monoclonal antibody (MAb) or anti $\beta$ type platelet-derived growth factor receptor (PDGFR-$\beta$) antibody (Ab) was injected, or splenectomy was performed in the rats which were then followed until 10 to 11 weeks posttransplantation. The 22 rats with chronic graft rejection showed increased CD8a-like antigen (probably Fas ligand) on the peripheral blood T cells. All the liver grafts had both necrosis and apoptosis. The liver graft apoptosis was indicated by histopathological abnormalities, and by DNA strand breaks and hemosiderin depositions in the cytoplasm. PDGFR-$\beta$ expression in the apoptotic liver graft was demonstrated immunohistochemically. Among the 17 rats injected with anti-CD2 MAb, CD2 signaling on host T cells was effectively suppressed by the injection of anti-CD2 MAb in 4 rats with better-surviving liver grafts. In these 4 rats, CD28 antigen on thymic lymphocytes was down-modulated and high numbers (136-233-positive cells per lobe) of the epithelial reticular cells with apoptotic lymphocytes were counted. Anti-PDGFR-$\beta$ Ab caused high pulmonary secretions of growth factors and reticular fibrosis in the lungs of 5 rats injected with the Ab. Anti-PDGFR-$\beta$ Ab injection reduced the host cell apoptosis in the lung and thymus, but did not prolong the survival of liver grafts. In the 9 rats with both splenectomy and anti-CD2 MAb injection, pulmonary apoptosis was induced with the 6-16% reductions of $CD4^+$ lymphocytes. Prolonged graft survival was observed in only one of the 9 rats. Anti-CD2 MAb was effective for prolonging the liver graft survival with suppressed CD28 antigen, but anti-PDGFR-$\beta$ Ab and splenectomy were not. ———— anti-CD2 MAb; anti-

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PDGFR-$\beta$ Ab; splenectomy; liver graft; apoptosis 

Graft rejection is produced by immunological reactions between graft and host. In graft rejection, graft and host cell deaths are divided into apoptosis and necrosis (Hayashi et al. 1997). Signaling via the T cell receptor (TCR)/CD3 complex in the presence of CD2 co-stimulatory signals induces antigen-specific T cell activation and prevents the T cells from undergoing apoptosis. Down-modulated CD2 signaling in allogeneic immune reactions is important for prolonging graft survival times after the grafted organ has been vascularized (Sido et al. 1998). CD28 antigen and TCR are reported to be codependent, and CD28 responses increase IL-2 production and render T cells resistant to apoptotic cell death (Okkenhaug and Rottapel 1998). Cytotoxic T cells induce apoptosis in their target cells. T-cell-rich large-B-cell lymphomas, which contain many $CD8^+$ cells, show increased lymphoma cell apoptosis (Felgar et al. 1998). Alloimmune injury leads to the expression of platelet-derived growth factor (PDGF) ligands in the graft vasculature (Lemström and Koskinen 1997). The expression of type $\beta$ PDGF receptor (PDGFR-$\beta$), which binds only PDGF-BB with high affinity, has been shown to be enhanced in human chronic pancreatitis (Ebert et al. 1998).

In this study, a piece of liver was engrafted on the host liver of 22 Lewis rats at 10 weeks of age. The effects of anti-CD2 monoclonal antibody (MAb), anti-PDGFR-$\beta$ antibody (Ab) and splenectomy on the graft survivals were examined in the rats transplanted with liver of the same strain. At 10–11 weeks posttransplantation, the apoptotic death of the graft hepatocytes was shown by the detection of degraded DNA in the cytoplasm. Strong expression of PDGFR-$\beta$ on the apoptotic hepatocytes was detected immunohistochemically. Among the 22 rats with liver grafts, the 4 rats which were injected with anti-CD2 MAb showed better survival of the liver grafts. The thymus of the 4 rats had suppressed lymphocyte expression of CD28 and increased apoptotic lymphocytes in epithelial reticular cells. Neither blockage of PDGFR-$\beta$ nor splenectomy was beneficial for the graft survival even though the liver grafts were vascularized and many new biliary ducts proliferated in contact with the host livers.

## Materials and Methods

### *Animals*

Lewis (LEW/Sea) rats were bred under controlled conditions in the Animal Center of Hamamatsu University School of Medicine. The mean body weights of the Lewis rats were 93 g in males and 74 g in females at the age of 5 weeks. Liver grafts were obtained from related 12-week-old Lewis females. The host rats were sacrificed at 10–11 weeks posttransplantation.

*Designs of the experiments*

Rats were divided into five groups: A, B, C, D and E. In groups A, B and D, liver transplantation was performed as follows. The anaesthetized rat was laid on its back. After abdominal skin and muscle incisions were made, the median or left lateral lobe was pushed out from the abdominal cavity. The liver graft, 3 mm in width and 8 mm in length, was attached with 2 stitches onto the surface of the host lobe.

Group A: Eight males received liver transplants at 10 weeks of age.

Group B: Nine males were splenectomized at 5 weeks of age and then received liver transplants at 10 weeks of age.

Group C: Seven females were splenectomized at 5 weeks of age.

In groups A, B and C, monoclonal mouse anti-rat CD2 equivalent of 12 $\mu$g per rat (Harlan Sera-lab Ltd., Crawley Down, Sussex, England) was injected subcutaneously, once a week for 4 weeks and then once a month for a month after liver transplants.

Group D: Five males received liver transplants at 10 weeks of age.

Group E: Six females were followed without any surgical treatment.

In groups D and E, anti-PDGFR-$\beta$ Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was injected subcutaneously. The anti-PDGFR-$\beta$ Ab was a goat polyclonal antibody made against a peptide corresponding to amino acids 1075–1094 of the PDGFR-$\beta$ precursor of mouse origin. When the rats were 5, 6 and 7 weeks of age, the anti-PDGFR-$\beta$ Ab was injected at doses of 5 $\mu$g, 17 $\mu$g and 27 $\mu$g, respectively, into the groups D and E rats. Only in group D, an additional 24 $\mu$g of anti-PDGFR-$\beta$ Ab was further injected once a week for 4 weeks after liver transplants.

*Flow cytometry (FCM) analyses*

The immunofluorescence bound to surface antigen on the lymphocytes separated from peripheral blood (PB) and thymus was analyzed using an EPICS(R) Profile II Analyzer (Coulter, Maiami, FL, USA). Red cells in the separated cells were lysed using the lysing solution of Ylem code LYS100 (YLEM SRL, Via Gramsci, Roma, Italy). One million PB cells were incubated separately with 0.5 $\mu$g of mouse IgG1, anti-rat CD4 MAb conjugated with fluorescein isothiocyanate (FITC) (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) and with 0.7 $\mu$g of mouse IgG1, FITC anti-rat CD8a MAb (Cedarlane Laboratories Limited) for 30 minutes at 4°C. Anti-rat CD8a MAb labels a T cell subset which mediates the suppression of antibody formation and the cytotoxic cell precursor. A complex of glycoproteins of Mr 34 000, 39 000 and 76 000 Da, which are the homologues of the human CD8 and mouse Ly-2, 3 antigens is recognized by the anti-CD8a MAb. One million thymic cells were incubated separately with 0.5 $\mu$g of the FITC anti-rat CD4 MAb described above and with 1 $\mu$g of mouse IgG1 anti-rat CD28

conjugated with FITC (Serotec Ltd., Kidlington, Oxford, England) for 30 minutes at 4°C. After fixation in 0.5% paraformaldehyde at 4°C overnight, the FITC-labelled cells were washed three times with phosphate buffered saline (PBS). The numbers of lymphocytes stained with FITC were counted to determine positive cell %.

*Apoptotic analyses*

The host liver, liver graft, thymus and lung of group A, B and D rats were fixed in 20% formalin, embedded in paraffin, and cut into sections. The liver graft sections were stained with hematoxlin-eosin (H-E) and with Berlinerblau. Programmed cell death was assessed in the paraffin-embedded tissue sections of the liver graft, thymus and lung. The ApopDETEK Cell Death Assay System and the Simply Sensitive Horseradish Peroxidase-DAB in Situ Detection System (Enzo Diagnostic, Inc., Famingdale, NY, USA) were used for the apoptotic analyses. Biotin-labeled nucleotide, Bio-16-dUTP, was incorporated into the 3′-OH termini in the fragmented DNA by terminal deoxynucleotide transferase activity. The Bio-16-dUTP incorporated onto the fragmented DNA was visualized using the streptavidin-horseradish peroxidase-diaminobenzidine-hematoxylin reaction. The positive complex was observed as a reddish precipitate. Genomic DNA was extracted from the livers of groups A, B, D and E rats and control males, and from the PB mononuclear cells of group A rats. For the DNA extraction, a QIAamp kit (QIAGEN Inc., Valencia, CA, USA) was used. The primers used for polymerase chain reaction (PCR) analysis of the bcl-2 gene were 5′ primer: 5′-CACCCCTGGCATCTTCTCCTTC-3′ and 3′ primer: 5′-CACAATCCTCCCCCAGTTCACC-3′ (Nagoya Katayama Chemical Co., Ltd., Nagoya) (Yamamoto 1998). Ampli Taq DNA polymerase (Perkin Elmer, Branchburg, NJ, USA) was used for the amplification of the DNA. PCR consisted of 30 cycles of denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute and extension at 72°C for 2 minutes. The DNA size marker used was GeneRuler 100 bp DNA Ladder (Fermentas Ltd., Graiciuno, Vilnius, Lithuania).

*Immunohistochemical analyses of PDGFR-β and interleukin II receptor (IL-2R) expression on liver grafts, and electron microscopic analysis of lung*

PDGFR-β and IL-2R expression on apoptotic grafts was examined using the liver graft sections of group A and B rats. The liver graft section of a group B rat was incubated with 6 μg of goat anti-mouse PDGFR-β Ab (Santa Cruz Biotechnology, Inc.) at room temperature for 30 minutes. The liver graft section of a group A rat was incubated with 15 μg of mouse anti-rat IL-2R MAb (Serotec Ltd., Kidlington, Oxford, England) at room temperature for 30 minutes. Ten micrograms of FITC-conjugated anti-mouse IgG1 were added to both of the sections and further incubated at 37°C for 10 minutes. The slides were washed with PBS and counter-stained with hematoxylin. The FITC-labeled sections

were observed with a laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA, USA). The lung of a group D rat which was injected with anti-mouse PDGFR-$\beta$ Ab was fixed with 2% glutaraldehyde for 2 hours and postfixed with 1% osmium tetroxide. The pieces were embedded with epoxy-resin, sectioned and stained with uranyl acetate-lead citrate. They were observed using a JEM 1220 transmission electron microscope (JEOL, Tokyo).

## Results

Table 1 shows the results of FCM analyses. The numbers of PB and thymic CD4-positive (+) lymphocytes were not increased in any of the rats grafted with syngeneic liver. In splenectomized group B rats, there were 6% fewer $CD4^+$ cells in PB and 16% fewer $CD4^+$ cells in the thymus than in control rats. The FCM profile of anti-CD8a MAb-stained cells contained three peaks. The $CD8a^+$ cell peak was shifted farthest to the right. The $CD8a^+$ cell content ranged from $22\pm4\%$ to $26\pm3\%$. The other two peaks contained inherently CD8a-negative cells. The proportional of cells in the middle peak changed in the rats with liver grafts. In groups A, B and D rats with liver graft rejection, the middle peak became 14–20% higher than that in control rats. These rats fell into two groups according to the proportion of $CD28^+$ cells in the thymus. The high % group contained 74 to $94\pm4\%$ of $CD28^+$ cells and the low % group contained $27\pm2$ to $56\pm5\%$ of $CD28^+$ cells. Control rats belonged to the low % group of CD28. The 3 rats of group A with $56\pm5\%$ of $CD28^+$ thymic cells showed better graft survival than the other rats of group A. One rat of group B with 39% $CD28^+$ thymic cells also

Table 1. *The % of $CD4^+$, $CD8a^+$ and $CD28^+$ cells counted in the PB and thymus*

| Exp. group | Sex | Total rats ($n$) | % positive cells in PB | | | % positive cells in thymus | |
|---|---|---|---|---|---|---|---|
| | | | CD4 (Mean±S.D.) | CD8a like (M±S.D.) | CD8a (M±S.D.) | CD4 (M±S.D.) | CD28 (M±S.D.) |
| A[a] | M | 8 | 66±5 | 66± 4 | 25±3 | 84±6 | 92± 5($n$=5)<br>56± 5($n$=3) |
| B[b] | M | 9 | 57±7 | 62± 6 | 26±3 | 74±9 | 94± 4($n$=4)<br>42± 5($n$=5) |
| C[c] | F | 7 | NT | 48±15 | 26±2 | NT | 75± 4($n$=2)<br>48± 8($n$=5) |
| D[d] | M | 5 | 65±4 | 60±10 | 23±5 | 91±1 | 44± 5 |
| E[e] | F | 6 | 68±3 | 45± 7 | 23±4 | NT | 74 ($n$=1)<br>35± 6($n$=5) |
| Control | M | 8 | 63±5 | 46±16 | 22±4 | 90±5 | 48±15 |
| Control | F | 5 | 66±2 | 52± 6 | 23±3 | 85±5 | 27± 2 |

[a] Transplant.+anti-CD2 MAb. [b] Splenect.+transplant.+anti-CD2 MAb. [c] Splenect.+anti-CD2 MAb. [d] Transplant.+anti-PDGFR-$\beta$ Ab. [e] Anti-PDGFR-$\beta$ Ab. NT, not tested.

TABLE 2. *The results of apoptotic cell death assays in the males with liver grafts*

| Exp. group | Graft hepatocytes | | | Thymus |
|---|---|---|---|---|
| | Size of graft mass | Advanced apoptosis | Fe-deposition | Apoptotic ER cell number per one lobe |
| A | Middle | + | + | NT |
| | Middle | + | + | 233 |
| | Middle | + | + | 136 |
| B | Small | + | + | 44 |
| | Middle | + | + | 189 |
| D | Small | + | + | 28 |
| | Small | + | + | 40 |

ER, Epithelial reticular cells of the cortical zone; NT, not tested.

showed a better graft survival than the other rats of group B.

The rejected liver grafts were necrotic and apoptotic. The liver grafts were accepted once and then rejected by 10–11 weeks posttransplantation. The apoptotic graft hepatocytes in contact with the host liver remained fairly intact. Most residual apoptotic hepatocytes had a smaller nucleus in a scantier cytoplasm than normal hepatocytes. However, some of the apoptotic hepatocytes had multiple nuclei in a large fused cytoplasm. There was marked angiogenesis and cholangiogenesis where the liver graft was in contact with the host liver. Many lymphocytes were infiltrated in the liver grafts. Table 2 summarizes the results of the apoptotic cell death assays. Among the 7 rats analyzed with the Apop-DETEK Cell Death Assay System, 4 rats had better graft survival at 10–11 weeks posttransplantation. A liver graft with advanced apoptosis of hepatocytes is shown in Fig. 1. Deoxynucleotides were incorporated into the 3′-OH termini of the fragmented DNA, leading to many peroxidase-positive granules of reddish color, mostly in the cytoplasm. Many of the host hepatocyte nuclei were stained brown with the peroxidase stain, but were not apoptotic. Hemosiderin deposits in the apoptotic hepatocyte cytoplasm were shown by Berlinerblau staining. Apoptotic epithelial reticular cells that had the horseradish peroxidase-DAB positive granules were counted in the cortical zone of one thymic lobe. Apoptotic epithelial reticular cells had apoptotic lymphocytes. Two rats of group A and one rat of group B, all of which had better-persisting grafts, had numbers of apoptotic lymphocytes in epithelial reticular cells. Apoptotic epithelial reticular cells were counted to be 136–233 per lobe. The other 3 rats, all of which had small graft masses showed small numbers of apoptotic epithelial reticular cells (28–44 per lobe). The numbers of the epithelial reticular cells with apoptotic lymphocytes were small in the 3 rats. One rat of group B with the 44 apoptotic cells per lobe was found to have 99% $CD28^+$ lymphocytes in the thymus. Fig. 2 shows the bcl-2 gene fragment amplified by PCR from the DNA extracted from the

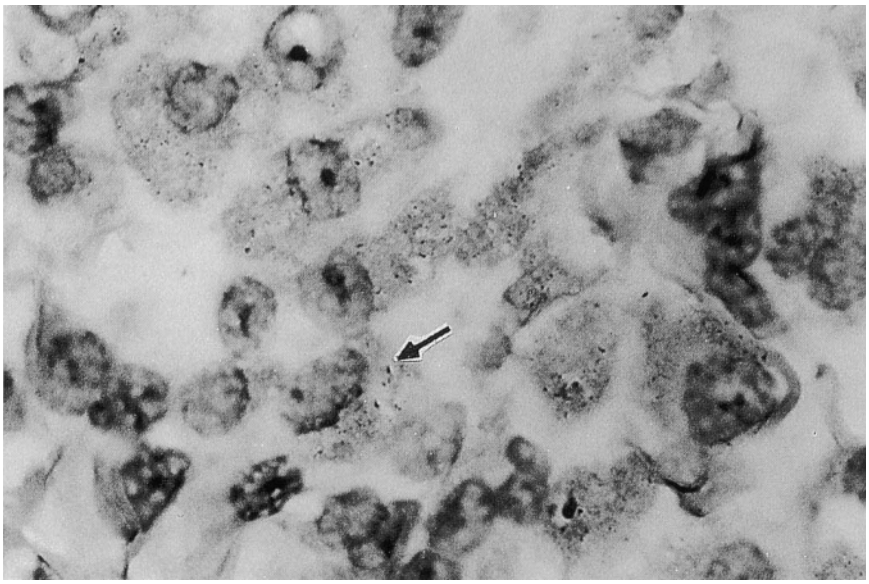

Fig. 1. The liver graft of a group B rat. The apoptotic hepatocytes were incorporated biotin-labeled nucleic acid, Bio-16-dUTP, onto the 3′-OH termini in the breakdown DNA, which were stained with the Simply Sensitive Horseradish Peroxidase-DAB in Situ Detection System. In the cytoplasm, many small reddish granules were recognized as shown by an arrow. (ApopDETEK-Streptavidin-peroxidase-hematoxylin stain, ×1000).

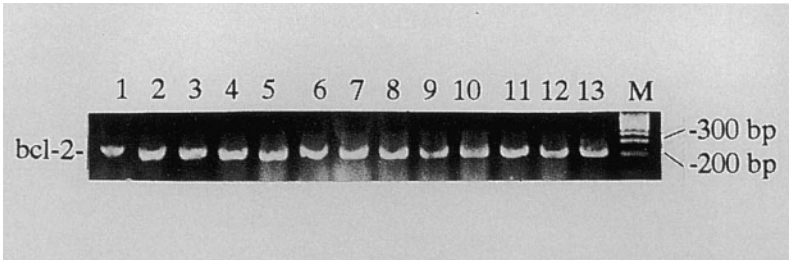


Fig. 2. PCR-amplified bcl-2 gene-specific fragments. The DNA was extracted from the host livers of groups B (lane Nos. 1–5) and D (lane Nos. 6–10), and control males (lane Nos. 11–13). The size of the bcl-2 gene-specific product was 200 bp.

hepatocytes of groups B and D rats, and control males. The size of the specific product was 200 bp. The bcl-2 gene fragment was also identified in the hepatocytes of groups A and E, and the PB lymphocytes of group A.

Fig. 3 shows apoptotic hepatocytes stained with anti-PDGFR-$\beta$ Ab, which were from the liver graft of a group B rat. The advanced apoptotic hepatocytes of the graft expressed PDGFR-$\beta$ on their surface. IL-2R expression on the apoptotic hepatocytes was also observed in the graft of a group A rat. Fig. 4 shows the lung of a group D rat that was injected with anti-PDGFR-$\beta$ Ab. Electron microphotograph of the lung of a group D rat indicated that active secretions occurred in the alveolar epithelial cells and endothelial cells. Reticular fibers were proliferated in the lung. Although the lungs of two splenectomized rats of group B contained 15–28 apoptotic pulmonary cells per the view-field

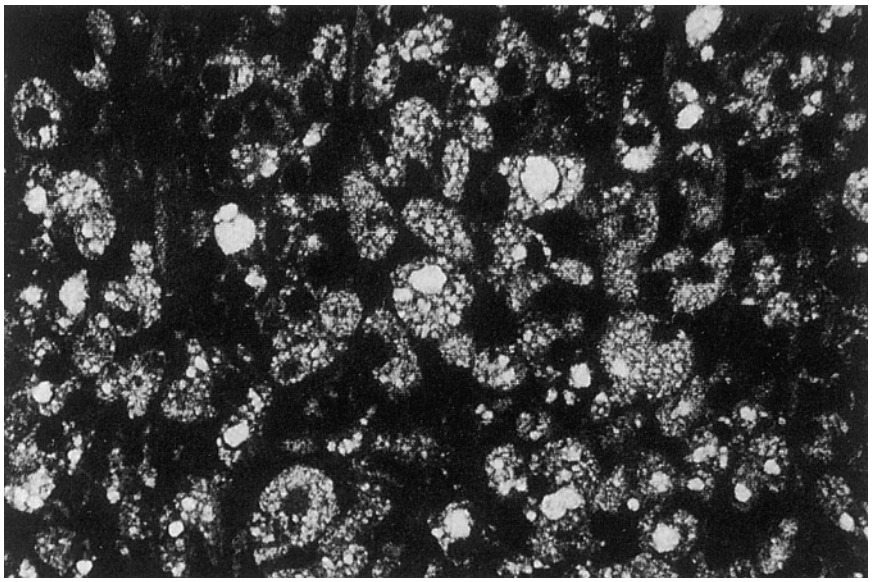

Fig. 3. The liver graft of a group B rat. The apoptotic hepatocytes were stained positively with anti-PDGFR-$\beta$ Ab, indicating expression of PDGFR-$\beta$ on apoptotic hepatocytes (Immunofluorescein staining).

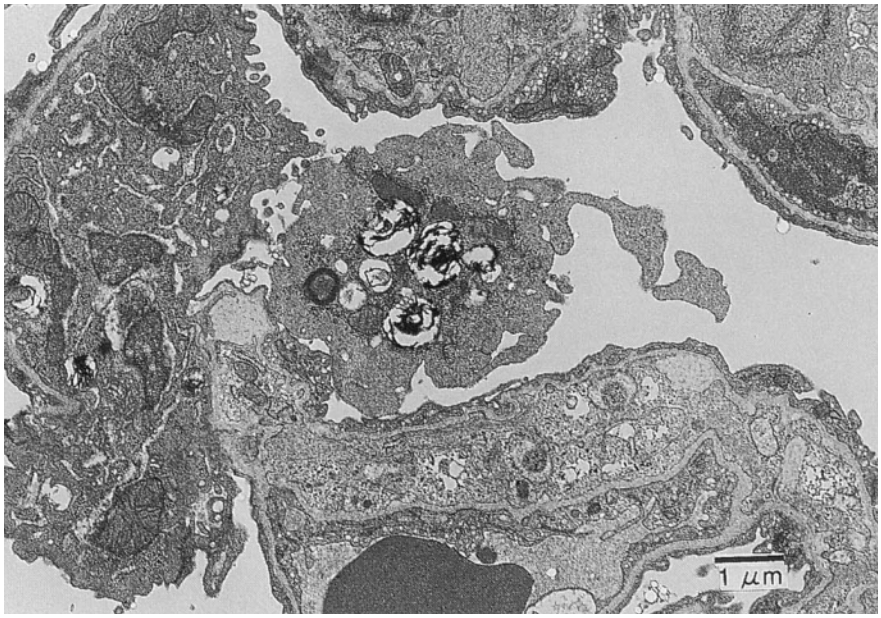


Fig. 4. Three alveolar epithelial cells (type II) appear here. They are rich in microvilli and granular endoplasmic reticulum (GER). One of the cells shows a thick lipid layer and the other two cells have flat surfaces. Under the alveolar basement membrane, reticular fibrosis is recognized. A lot of small vacuoles are present both in squamous alveolar cells (type I) on the upper side and in endothelial cells on the under side (Uranyl acetate-lead citrate double stain, $\times 12\,000$).

($\times 125$) of the light microscope, in the two rats of group D, 0–1 apoptotic cells were counted in the same microscopic field. Thus, apoptotic changes of the host lung and thymus were suppressed in the rats of group D, which were injected with anti-PDGFR-$\beta$ Ab, although general conditions of the group D rats were weaker

than those of the groups A and B rats.

## Discussion

Graft hepatocytes undergo apoptosis and necrosis in the chronic rejective reactions which are caused by the some mismatchings of minor histocompatibility antigens among LEW/Sea strain rats (Hayashi et al. 1997; Yamamoto et al. 1998). DNA strand breaks and hemosiderin depositions were found in the cytoplasm of the apoptotic graft hepatocytes. Multi-nuclear fused giant hepatocytes were also observed in the apoptotic liver. As shown in this study, at the most advanced stage, cellular DNA strand fragmentation was detected in the cytoplasm of apoptotic cells (Gorczyca et al. 1993). The bcl-2 gene, which encodes a general inhibitor of apoptosis, was amplified from host liver DNA by PCR. The liver bcl-2 gene appears to protect the non-apoptotic host liver from apoptosis. Recently, it was indicated that cytotoxic $CD8^+$ T cells might use Fas ligand (FasL) or tumor necrosis factor (TNF) to mediate bystander killing (Smyth and Sedgwick 1998). FasL-positive cytotoxic T cells proliferate better in response to allogeneic stimulation than do FasL-negative cytotoxic T cells (Suzuki and Fink 1998). In the FCM analyses of this study, the % $CD8a^+$ cells was not increased in the rats with graft liver rejection, but the antigen, probably the FasL cross-reacted with anti-CD8a MAb, was increased in the rats with graft liver rejection. Expression of FasL not only on $CD8a^+$ T cells but also on CD8a-negativeT cells might be occurring during the bystander killing of the liver grafts.

Anti-CD2 MAb was injected to prolong the liver graft survival in the rats grafted with a piece of syngeneic liver. When the lymphocytes of mesenteric lymphnodi were incubated in vitro with anti-CD2 MAb at 37°C for 30 minutes, in 3 of the 7 males, the lymphocytes lost 10% of CD28 expression (data not shown). The beneficial effects of anti-CD2 MAb must be shown by the suppression of not only CD2 but also CD28 on T cells. Among the 17 rats injected with anti-CD2 MAb, the 4 rats with a low proprtion of $CD28^+$ thymic lymphocytes showed better liver graft survival than the other rats. The numbers of apoptotic epithelial reticular cells were high in the rat thymus where there was a low proportion of $CD28^+$ lymphocytes. This reflected the fact that thymic lymphocytes died more rapidly in the thymuses of the 4 rats with better graft survival. CD28-negative thymic lymphocytes could not provide a co-stimulatory signal to achieve optimal T cell activation. The beneficial in vivo effects of anti-CD2 MAb were shown by the suppression of CD28 antigen on thymic T cells. Only 1 of the 9 splenectomized rats injected with anti-CD2 MAb was among the 4 rats with better graft survival. As the splenectomized rats had 16% fewer $CD4^+$ cells in the thymus, pulmonary cell apoptosis and graft liver infection might become bad factors. In the 4 splenectomized rats with a low proportion of $CD28^+$ thymic cells, liver graft survival was not improved.

The lungs of rats injected with anti-PDGFR-$\beta$ Ab might have actively

secreted some growth factors from the pulmonary epithelial and endothelial cells, which resulted in reticular fibrosis but not in apoptosis. Alveolar macrophages, mesenchymal cells, endothelial cells and platelets are known to be sources of platelet-derived growth factor (PDGF). Binding of the PDGF BB chain to the receptor induces dimerization and autophosphorylation of the PDGFR-$\beta$ at specific tyrosine residues. Phosphorylated PDGFR-$\beta$ binds a large variety of SH2 proteins, such as phosphatidylinositol 3′-kinase, SHP-2, phospholipase C$\gamma$1, Ras GTPase-activating protein and Src kinase family members. Recently, it was reported that phosphorylated PDGFR-$\beta$ stimulated protein kinase D (PKD) and subsequently, PKC (Lint et al. 1998). The blockage of PDGFR-$\beta$ phosphorylation should stop the activation of PKC. TCR- and CD28-mediated changes were blocked in the thymuses of rats injected with anti-PDGFR-$\beta$ Ab, because the Ab caused CD28 expression to be down-regulated in the thymus. The protooncogene Vav, which regulates peptide-specific apoptosis in the thymus, also seemed to be down-modulated, because the numbers of apoptotic reticular epithelial cells were small in the thymuses of rats injected with anti-PDGFR-$\beta$ Ab (Kong et al. 1998). The protective effects of anti-PDGFR-$\beta$ Ab against host cell apoptosis did not provide effective reactions to the liver gaft. On the contrary, the rats injected with anti-PDGFR-$\beta$ Ab were weaker than the rats injected with anti-CD2 Ab because of suppressed PDGFR phosphorylation in the host cells.

PDGF may have a helpful role in promoting graft vasculature and beneficially may affect graft survival (Lemström and Koskinen 1997). PDGFR expression on endothelial cells is important in cell migration and proliferation. To maintain the cell activation, the PDGFR-$\beta$ must be expressed strongly on the apoptotic graft hepatocytes at the last stage of apoptosis, and strong PKC activation must be triggered in the apoptotic hepatocytes.

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