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Renal or Liver Apoptotic Changes Triggered by Inoculation of Homogenic T Cells and Ensuring High IL-2R Expression on T Cells: A Comparison with Early Graft-versus-Host Reaction (GvHR)

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Summary. **Homogenic T cells were injected into the renal subcapsule of 19 Lewis rats and the liver subcapsule of 8 Lewis rats to examine apoptotic changes induced by T cells. Resulting phenomena were compared with graft-versus-host reaction (GvHR) induced in 5 F_1 (DAxLewis) male rats by the injection of DA female lymphocytes. The positive cell numbers of interleukin II receptor (IL-2R), CD18 or CD5 antigen were counted on the peripheral and splenic lymphocytes. The proportions of peripheral IL-2R ($\alpha\beta$)-positive cells ranged from $3\pm2\%$ to $12\pm5\%$ in 23 of the 27 Lewis rats. Splenic IL-2R-positive cells ranged from 10% to 25% in all 27 rats. Based on the numbers of CD5-positive splenic lymphocytes, 20% to 100% of splenic T cells were judged to express IL-2R. In three of the remaining four rats, peripheral IL-2R ($\alpha\beta$)-positive cells ranged from 47% to 85%. One of the four remaining rats showed 94% expression of IL-2R ($\alpha\beta\gamma$) on the peripheral lymphocytes: IL-2R ($\alpha\beta\gamma$)-positive lymphocytes of the rat also expressed strongly CD18 and CD5 antigens. In the F_1 rats with early GvHR, IL-2R expression was observed on $13\pm2\%$ of peripheral lymphocytes and $59\pm5\%$ of splenic T lymphocytes, while IL-2R-positive cell % of control F_1 males were 15 ± 7 and 55 ± 9, respectively. Renal tubular disappearance due to apoptosis and glomerular accumulation were found in the renal cortex of the female rat with 94% IL-2R-positive cells. The same renal lesions were also observed in 6 other rats. Abundant Golgi's complexes were shown in a glomerular epithelial cell. In the T cell-inoculated liver, only focal lymphocyte proliferation was found abnormally around the sinusoid without the other apoptotic changes. IL-2R was strongly expressed on hepatocytes surrounding the focal lymphocyte proliferation. In the plasma of the F_1 rats with GvHR, secreted humoral factors which activated monocytes but not neutrophils were indicated by the phagocytic functions of apoptotic cells, *in vitro*.**

It was concluded that IL-2R on T cells was activated by homogenic cell reactions such as apoptosis, but not by heterogenic donor cell reactions such as GvHR, in which antiapoptotic interferon (IFN)-like receptors were considered to be activated.

Key words—apoptosis, IL-2R, GvHR, Golgi's complex, rat.

INTRODUCTION

Interleukin II receptors (IL-2R) are expressed primarily on T cells but have also been observed on B cells, macrophages and NK cells. Coexpression of an α chain (p55, Tac) and a β chain (p75) is found on 1% to 6% of normal peripheral lymphocytes.[1] The IL-2R β chain contains at least two cytoplasmic portions: One is a serin rich region critical for mitogenic signaling and the other is an acidic region responsible for interaction with protein tyrosine kinases, p56.[lck] The binding of IL-2 to IL-2R on T cells promotes cytotoxic activity and the expression of intercellular adhesion molecule-1 (ICAM-1). Immunological reactions mediated by cytotoxic T cells result in target cell apoptosis (programmed cell death).[2] ICAM-1 is also a key mediator of apoptotic changes.[3] Apoptotic and regenerative changes have been investigated thoroughly in the kidney.[4-6]

This study indicated a correlation between apoptotic changes and high IL-2R expression on

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peripheral T cells. The apoptosis was triggered by homogenic T cell injection in renal or hepatic subcapsules. Renal tubular apoptosis, resistant reactions of glomerular epithelial cells and focal lymphocyte proliferations in hepatocytes were shown. Graft-versus-host reaction (GvHR), which rather belonged to antiapoptotic reactions was compared with the apoptotic changes triggered by homogenic T cells.

MATERIALS AND METHODS

Rats: Lewis (LEW/Sea) rats were bred in a colony for 1.5 years at the Hamamatsu University School of Medicine, Japan. DA (DA/Slc) rats were purchased from Japan SLC, Inc. and bred in the same facility. Lewis rats and F_1 (DAxLewis) rats were used as hosts. Experiments A to F constituted injections of ectopic Lewis T cells into the Lewis rat kidney (A-D) or liver (E-F). Only experiment G was planned to induce graft-versus-host disease (GvHD) in F_1 (DAxLewis) rats. Four to six rats were used in each experiment: 11 to 12 weeks old Lewis rats were used in experiments A and D; 7 to 8 weeks old Lewis rats in experiments B, E and F; 4 weeks old Lewis rats in experiment C, and 5 weeks old F_1 (DAxLewis) rats in experiment G. Lewis ($RT1^l$) and DA ($RT1^a$) rats were used as donors.

Cell engraftment procedures: For experiments A, B, C and D, male and female Lewis rats were anesthetized with diethylether and the left kidney was pulled out via the dorso-ventral incision.[7] Thymus and bone marrow cells ($1\text{-}2\times10^8$ cells/rat) obtained from Lewis rats were injected into the subcapsule or the superficial cortex of the kidney. For experiments E and F, male and female Lewis rats were anesthetized with diethylether and the left lateral lobe of the liver was pushed out via the abdominal incision by hand.[7] Thymus cells ($3\text{-}8\times10^8$/rat) obtained from male Lewis rats were injected into the liver. Rats in experiments A, B, C and D were followed for 139 to 144 days, while rats in experiments E and F were observed for 51 days. For experiment G, male F_1 (DAxLewis) rats were injected subcutaneously twice with $3\text{-}6\times10^7$ Lewis ($RT1^l$) cells separated from the spleen, thymus and lymph nodes of male Lewis rats at 6-day intervals. On day 62 after the second immunization, the rats received intravenous injections of 2.6×10^8 splenic cells, 1.0×10^8 lymphnode cells, and 5.6×10^6 peripheral mononuclear cells (PMNC), which were obtained from DA ($RT1^a$) female rats. On day 9 after the third immunization, experiment G rats were sacrificed by aspirating the arterial blood.

Fluorescent cell sorting: Free-floating lymphocytes labeled with fluorescein isothiocyanate (FITC)-conjugated antibody were counted automatically in the flow cytometer of an EPICS(R) Profile II Analyzer (Coulter, USA). PMNCs and splenic mononuclear cells (SMNC) were isolated from rats at sacrifice. T cells were separated from SMNCs of F_1 rats using T Lymphokwik (One Lambda, Inc., USA). The expression of antigens on lymphocytes was examined using 3 mouse IgG1 antibodies as unconjugated reagents. Forty μl (9.18 μg) of the mAb to rat IL-2R, 20 μl (0.2 μg) of the Ab to rat CD18 (ICAM-1) or 25 μl (1.15 μg) of the mAb to rat thymocytes and T cells (CD5) (Serotec, England) was added to 10^6 lymphocytes in 50 μl phosphate-buffered saline (PBS). First, the preparations were incubated at room temperature for 40 min. Anti-mouse IgG1-FITC (10 μg/μl protein) (The Binding Site Ltd, England) was then added as the second antibody. The preparations were maintained at 4℃ for 30 min. The reactions were stopped by the addition of 1% paraformaldehyde. After overnight incubation at 4℃, cells were washed 3 times with PBS. IL-2R ($\alpha\beta$)-positive cells were counted as IL-2R-positive cells. Cells showing strong and weak staining for CD18 were counted separately in F_1 rats.

Histopathological and immunofluorescent analysis: The kidney and liver were fixed in 20% formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin (H-E) and Prussian blue. In experiment D, only section from the No. 2 rat was stained with FITC rabbit anti-rat-thrombocyte Ab (Inter-Cell Technologies Inc., USA). In experiment E, two liver sections of No. 2 and No. 3 rats were stained with both the mAb to IL-2R and anti-mouse IgG1-FITC. The sections labeled with FITC were further counterstained with hematoxylin and observed under a fluorescent microscope (Karl-Zeiss, Germany). The renal cortex of No. 5 rat in experiment A was fixed with 2% glutaraldehyde for 2 hr and postfixed with 1% osmium tetroxide. Pieces of the cortex were embedded with epoxy resin. Sections were examined with a transmission electron microscope (JEOL 100CX).

Apoptotic cell phagocytosis: Leukocytes were obtained from 7 ml of whole blood obtained from 1 male Lewis rat and 1 female Lewis rat sacrificed at the age of 3 months. PMNCs were separated from the male blood using a Ficoll-Paque gradient (Pharmacia Biotech, Sweden). The PMNCs were fixed in methanol for 5 min and stained with hematoxylin for 5 min. The stained cells were washed several times with PBS and water and then suspended in 300 μl of PBS. Leukocyte-rich blood (350 μl) was prepared from the female blood. Two μg of chemotactic peptide, N-Formyl-L-Met-L-Leu-L-Phe-OH (fMLP), (Bachem Inc., USA) in 20 μl of PBS was added to 50 μl of

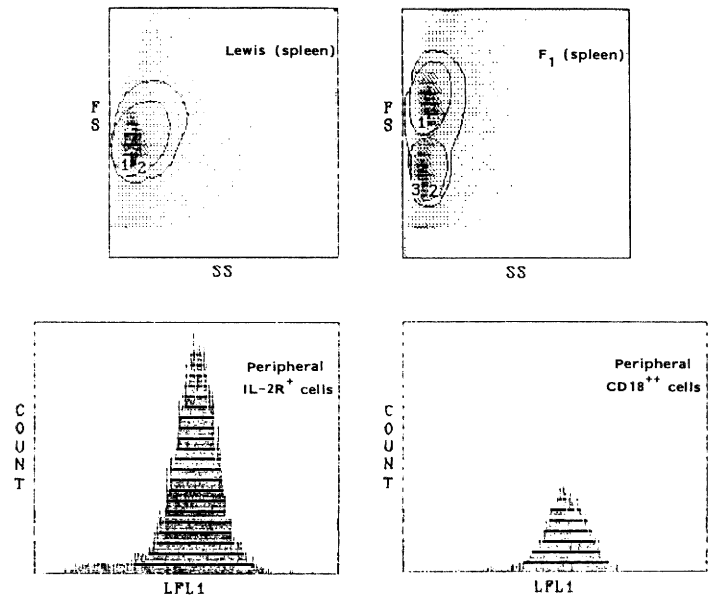

Fig. 1. Fluorescent cell sorting results of lymphocytes labeled with FITC-conjugated antibody. Upper panel shows gating bitmaps of splenic lymphocytes from Lewis and F_1 (DAxLewis) rats. Gating bitmaps of splenic lymphocytes from F_1 rats are separated into two bitmaps, that is, upper bitmap 1 and lower bitmap 3. Lower panel presents peripheral lymphocytic histograms in No. 2 rat in experiment D.

plasma from male experiment G rat and control F_1 male rats. The fixed $PMNC_s$ (8 μl) and leukocyte-rich blood (8 μl) from Lewis rats were added to 70 μl of the mixture of plasma a & fMLP and 50 μl of simple plasma, and incubated for 2 hr at 37℃. After smearing, the smeared sildes were stained with May-Gruenwald-Giemsa solution. Monocytic and neutrophil phagocytes were counted separately on the smears to calculate the percent pagocytosis of each cell. Significant differences of the percent phagocytosis were also examined between them.

RESULTS

A nodule of diameter about 0.5 to 1.0 cm was found at the incised sites of Nos. 1, 2 and 3 rats in experiment C and Nos. 1 and 2 rats in experiment D 26 to 42 days after the injection of T cells into the kidney. The nodules removed from the rats were pustules formed in the subepimysium of the incised muscle. The same pustule was removed from the subepimysium of the abdominal muscle of No. 4 rat in experiment F 9 days after the injection of T cells. All the pustules had a hard capsule. Rats in experiment G showed early signs of GvHD 9 days after the third injection, together with splenomegaly. Among the animals, GvHD signs and splenomegaly were more severe in Nos. 1 and 3 rats than Nos. 2, 4 and 5 rats.

Fig. 1 demonstrates the results of fluorescent cell sorting. The lower panel shows the histograms of peripheral lymphocytes measured in No. 2 rat in experiment D. These histograms show that 94% of lymphocytes were IL-2R ($\alpha\beta\gamma$) positive and that 96% of the lymphocytes expressed large amounts of the β2 integrin (CD18). Positivity for CD5 antigen observed in 95% of the lymphocytes. The IL-2R-positive cell populations on peripheral lymphocytes ranged from $3\pm2\%$ to $12\pm5\%$ in 23 of the 27 Lewis rats (Table 1). 85% of peripheral lymphocytes were IL-2R ($\alpha\beta$) positive in the No. 3 rat in experiment C and No. 1 rat in experiment D, in that a pustule formation was found in the subepimysium of the incised muscle. In the No. 1 rat in experiment E, 47% of peripheral lymphocytes were IL-2R ($\alpha\beta$) positive. Expression of IL-2R on splenic T lymphocytes was stronger in the treated rats than in the control rats. IL-2R was expressed on 70 to 100% of CD5-positive splenic T cells in experiments C, E and F rats. Expressions of IL-2R and strong CD18 antigens were not increased on the peripheral lymphocytes in F_1 rats with GvHD, as compared with normal F_1 males (Table 2). IL-2R

expression on splenic T cells was only 4% higher in F_1 rats with GvHD than in normal F_1 males.

As shown in Fig. 2, the accumulation of glomeruli and disappearance of tubules around them were observed in the kidney of the No. 2 rat in experiment D with 94% IL-2R-positive cells. Apoptotic tubules that had decreased in size were rounded up and their desmosomal attachments to neighbouring cells had disappeared. Some tubules had two nuclei. Many of the glomeruli were atrophic. Hemosiderin deposition, which was stained with Prussian blue, was present in some tubules. Only the red cells in the apoptotic area were stained strongly with the anti-rat-thrombocyte antibody. The same renal lesion was also observed in the renal cortex of the No. 4 rat in experiment D and in the norrow areas of Nos. 4 and 5 rats in experiment A, Nos. 2 and 3 rats in experiment B and No. 3 rat in experiment C. Fig. 3 shows the visceral epithelial cells of the glomerulus to be rich in Golgi's complex and polysomes. In Exp. E rats, hepatocytes with relatively monotonous nucleus of about a 7.5 μm diameter were found. Focal, but small, lymphocyte

Table 1. IL-2R-positive cells and CD5-positive cells on peripheral and splenic lymphocytes of Lewis rats injected with Lewis thymic T cells into the kidney or the liver

Exp. No.-Rat No. (Total No.)		Sex	Organ	Periphery	Spleen	
				IL-2R$^+$ cells (%)	IL-2R$^+$ cells (%)	CD5$^+$ cells (%)
A-1, 2, 3, 4, 5	(n=5)	M	Kidney	3±2	10±7	ND
B-1, 2, 3, 4, 5, 6	(n=6)	M	Kidney	8±2	12±7	57±7
C-1, 2, 4	(n=3)	M	Kidney	6±2	26±4	28±3
-3	(n=1)	M	Kdney	85	19	22
D-1	(n=1)	F	Kidney	85	14	ND
-2	(n=1)	F	Kidney	94*	12	ND
-3	(n=1)	F	Kidney	ND	ND	ND
-4	(n=1)	F	Kidney	6	9	ND
E-1	(n=1)	M	Liver	47	26	20
-2, 3, 4	(n=3)	M	Liver	12±5	22±3	22±1
F-1, 2, 3, 4	(n=4)	F	Liver	10±5	24±7	33±6
Cont-1, 2, 3, 4	(n=4)	M	ND	1±0	4±2	51±5
Cont-1, 2, 3, 4	(n=4)	F	ND	2±1	7±1	56±9

Data are the mean±SD.
*95% of lymphocytes were CD5-positive and 96% were CD18-strongly positive.

Table 2. IL-2R-positive cells and cells showing strong expression of β2 integrin (CD18) on peripheral lymphocytes and splenic T cells from F_1 (DAxLewis) male rats with GvHD and F_1 control rats

Exp. No. (Sex)-Rat No. (Total No.)		IL-2R$^+$ cells (%)			CD18^{++} cells (%)		
		Bitmap 1*	Bitmap 3*	Total	Bitmap 1*	Bitmap 3*	Total
Peripheral lymphocytes:							
G (M)-1, 2, 3, 4, 5	(n=5)	7± 1	29± 5	13± 2	8± 2	24± 8	9± 3
Cont (M)-1, 2, 3, 4	(n=4)	6± 2	36±13	15± 7	11± 3	32± 8	12± 2
Splenic T cells:							
G (M)**-1, 2, 3, 4, 5	(n=5)	30± 4	67± 6	59± 5	67±12	81± 3	83± 3
Cont (M)-1, 2, 3, 4	(n=4)	15±10	63±10	55± 9	62±14	79± 8	79± 8

Data are the mean±SD.
*See Fig. 1 (upper panel).
**Histopathologically, splenic lymphocytes were remarkably activated.

proliferations were recognized around the sinusoids of experiment E and F rats. Hepatocytes around the the area of lymphocyte proliferation were positively stained with the mAb to IL-2R. The F_1 rats with GvHD showed massive lymphocyte infiltration in the portal spaces of the liver and remarkable lymphocyte activation in the spleen.

Table 3 indicates the results of the percent phagocytosis counted in monocytes and neutrophils. Although Lewis monocytic phagocytosis was slightly activated even in the plasma of normal F_1 rats, monocytic phagocytosis of apoptotic cells was 10% higher in the plasma of F_1 rats with GvHD than in the plasma of normal F_1 male rats ($p=0.02$). About 10 to 20% of Lewis neutrophils lost their phagocytal abilities in the plasma of all F_1 rats. The fMLP added to the plasma of rats with GvHD increased the percents of neutrophil phagocytosis from $15\pm2\%$ to $43\pm3\%$

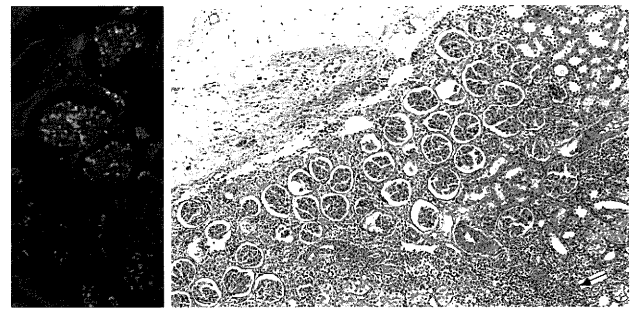

Fig. 2. The renal cortex of No. 2 rat in experiment D, in which ectopic T cells were injected. Both glomerular accumulation and tubular apoptosis are observed. Many atrophic glomeruli are present. Infiltrated lymphocytes and angiogenesis are detected at the margins. The arrow indicates arteriole clusters (H-E stain, ×40). The red cells receiving a proteolytic enzyme reaction are positively stained with FITC conjugated anti-thrombocyte antibody.

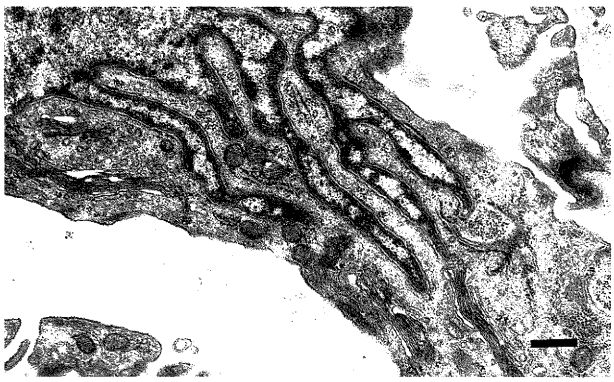

Fig. 3. Electron micrograph of a visceral epithelial cell from the glomerulus of No. 5 rat in experiment A. A convoluted nucleus with well-developed granular endoplasmic reticulum (ER) is seen. Four Golgi's complexes are present. The cytoplasm is rich in polysomes. Ribosomes are recognized in small mitochondria. The bar is 0.5 μm (uranyl acetate-lead citrate double stain, ×12,000).

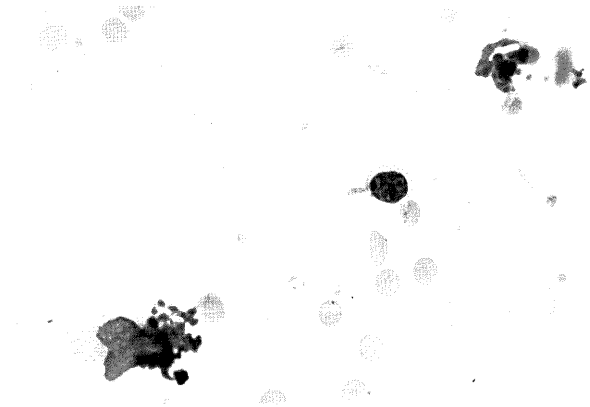

Fig. 4. Lewis leukocyte phagocytosis of Lewis apoptotic cells in the plasma of No. 1 rat in experiment G containing fMLP. Both monocytes and neutrophils actively phagocytize apoptotic nuclear fragments (May-Gruenwald-Giemsa stain, ×520).

Table 3. Lewis leukocytic phagocytosis of Lewis apoptotic cells in the plasma of F_1 (DAxLewis) male rats with GvHD and the plasma of normal F_1 rats

Used plasma Exp. No. (Total No.)	Sex	Monocytic phagocytosis Positive cells (%)	Neutrophil phagocytosis Positive cells (%)
G (n=5)	M	64±4*	15±2**
G (n=5) added fMLP	M	62±9	43±3**
Cont (n=5)	M	54±3*	21±4
Cont (n=5) added fMLP	M	56±8	39±8
Cont (n=4)	F	44±5	14±4
Lewis autoplasma (n=1)	F	47	34

Data are the mean ±SD.
Significant difference between them; *$p=0.02$, and **$p=0.001$.

($p=0.001$). Clusters of apoptotic nuclear fragments from the fixed Lewis PMNCs were recognized on the smeared slides. The monocytic and neutrophil phagocytoses of apoptotic bodies are shown in Fig. 4.

DISCUSSION

The present results showed that expressions of IL-2R and $\beta 2$ integrin on peripheral T lymphocytes enhanced immune reactions, leading to apoptosis. Glomerular accumulation due to tubular apoptosis at the site of cell injection could be observed in 7 of the 19 Lewis rats in which homogenic T cells were injected into the renal subcapsule. Massive angiogenesis and lymphocyte infiltration were found at the margins of apoptosis. In the rat, it is said that nephrogenesis ends by the 8th postnatal day.[8] However, in this study, judging from the histopathological findings of the kidney, it was concluded that the glomerular accumulation did not depend only on tubular disappearance, but also on angiogenesis triggered in the area. It has been noted that the glomeruli develop processed vasculature and the juxtaglomerular apparatus also arises from epithelial cells in the vascular crevice.[9] Glomerular formations which might have some structural defects occurred in the apoptosis because of ischemia. It was also postulated that ischemic changes based on inoculated cell mass caused tubular apoptosis. DNase 1, which causes apoptosis, has been detected in the basement membranes.[1,2,10] As kelley et al. reported that genetic defects in ICAM-1 and the anti-ICAM-1 antibody protected mice against renal injury,[3] it was suggested that the strong expression of $\beta 2$ integrin (ICAM-1) on lymphocytes found in this study might have been involved in tubular death. Activated matrix metalloproteinase (MMP) might degrade red cell membranes and expose otherwise hidden glycoprotein with a proteolytic reaction.[10] Those red cells cross-reacting with anti-thrombocyte antibody were actively destroyed in the area as shown by hemosiderin deposition stained with Prussian blue. Elevated IL-2R and $\beta 2$ integrin on peripheral T cells were accounted for by a relation with tubular apoptosis.

As tubular apoptosis was always associated with glomerular accumulation, glomerular resistance was also shown. As was expected, Golgi's complex was abundant in the visceral epithelial cells of the glomerulus. Enzymatically-inactive lysosomal enzymes are sorted in Golgi's complex.[11] The ability of Golgi's complex to create enzymatically-inactive forms of lysosomal proteins may protect cells from the hydrolytic activity. If secretory proteins were not transported to Golgi's vesicles, the rough endoplasmic reticulum (ER) would be denatured because of the acid pH. Metabolic acidosis leads to apoptosis.[12] In the Lewis liver inoculated homogenic T cells, small, focal lymphocyte proliferation was found. IL-2R was expressed on hepatocytes surrounding the lymphocyte proliferation. Generally, tyrosine-specific phosphorylation occurs very rapidly after IL-2R activation.[1] Thus, tyrosine aminotransferase activation is caused by the activation of IL-2R on the hepatocytes. Hepatic tyrosine aminotransferase converts tyrosine and tryptophan to acetyl CoA and then to glucose.[11] The up-regulation of ATPase induced by glycolysis protects hepatocytes from ischemic necrosis. Accordingly, the strong expression of IL-2R on hepatocytes protected the hepatocytes from necrosis. As hepatocytes are known to be rich in Golgi's complex, apoptotic changes have been also considered to be protected.

Monocytic phagocytosis of apoptotic cells was 10% greater in the plasma of F_1 rats with GvHD than in the control plasma. Monocyte chemoattractant protein (MCP) receptor and anti-inflammatory cytokine receptors may have been expressed on monocytic macrophages, which leads to cytoplasmic G-protein activation. Activated cytoplasmic G-proteins increase in free calcium and decrease in intracellular pH, which protects macrophages from apoptosis. The activated expressions of antiapoptotic interferon (IFN)-like receptors were also considered in the GvHD rats because of accelerated monocytic phagocytosis and remarkable B cell activations in the spleen.[1] On the other hand, neutrophil phagocytosis was decreased in the plasma of the rats with GvHD. The addition of fMLP *in vitro* restored neutrophil phagocytosis in the plasma of the rats with GvHD. The fMLP activates cytoplasmic phosphorylation and extracellular signal-regulated kinases (ERK)-2 in neutrophils.[13] Judging from apoptotic changes of neutrophils, fMLP was shown not to be secreted in the plasma of F_1 rats with GvHD.

Elevated levels of IL-2R and adhesion molecules on lymphocytes have been reported to be correlated with autoimmune reactions in patients with autoimmune disease.[14] In this study, high levels of IL-2R on peripheral T cells could be shown in the 2 rats with tubular apoptosis. T cell receptor (TCR) recognized autoantigens might result in the activation of cytoplasmic G-protein and IL-2 secretion. Increased secretion of IL-2 might lead to T cell dependent autoimmune reactions with a high expression of IL-2R.

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