以化學及放射線治療預防異體肢體移植後移植物-宿主病之研究

Effect of Chemotherapy and Irradiation Therapy on Graft-Versus-Host Disease in Allotransplantation of Rat Limb

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Abstract

The purpose of this study is to establish a simple surgical model of limb allotransplantation in order to study the nature of graft versus host disease. Two transplantation models were developed, which included, Osteo-myo-cutaneous (OMC) transplant model and the traditional full-limb transplant model. Brown Norway(BN) to Lewis(LEW) rats combination was used in the study. Leflunomide at the dose of 10mg/kg with cyclosporine (short term:30 days, Long term: 60 days) or FK506 (low-dose: 0.1 mg/kg, high-dose: 0.5 mg/kg) /kg) were given as immunosuppresants after transplantation. After stoppING the immunosuppression agents, clinical sign of GVHD or rejection of the grafts were observed and histologically examined using ED1 monoclonal antibodies in order to investigate the macrophage activity during GVHD. Finally, we compared the GVHD after limb allotransplantation with the GVHD after clinical bone marrow transplantation and P to Fl animal model in the references. As a result, only the traditional limb transplantation model developed GVHD, two of six rats showed clinical signs including sudden weight loss, poor spirit, ocular and nasal discharge, and dyspnea. Lung and lymphoid tissues (thymus and mesenteric lymph nodes) were the major target organs in pathology, which was different from GVHD after BMT. However, some rats in the OMC model, with high dose of FK506 showed the histological but not clinical evidence of GVHD. In the observation of graft rejection, the traditional limb grafts were more easily rejected than the OMC model. And high dose FK506 combined with leflunomide gave more protection than other immunosupression protocols. The macrophage activity increased during GVHD,which is similar to the changes during GVHD after BMT.

Introduction

Graft versus host disease (GVHD) was the major implication after bone marrow transplantation(BMT).(33) Although we understood more it's feature today, but most studies were still focus on the GVHD after bone marrow transplantation. GVHD must be a potential problem after vasculized bone marrow transplantation(VBMT) because the impact bone marrow environment could provide many immunocompetent cells.(35) Limb transplantation was a kind of VBMT therefore GVHD may be a complicating factor in future clinical limb transplantation. GVHD after limb allotransplantation was found occasionally in the study to rejection model after limb transplantation, it was always following stopping immunosuppressed agent.(3,4,36,37) In these studies they only mention the clinical signs and incidence but not compare the pathological and other feature of the GVHD after limb allotransplantation whit GVHD after bone marrow transplantation. These animal models were developed to study rejection after limb transplantation instead of GVHD. Because of complicated transplanted procedure and long immnosupressed duration, these animal models were not very convenient for study. (3,4,36) The purpose of this study is to establish a simple surgical model of limb allotransplantation in order to study the nature of GVHD.

Material and method

Adult inbred male Brown-Norway(BN)(RT1^n) and LEW(RT1^l) rats which weighed 250-300g were purchased from National Laboratory Animal Breeding and Research Center in Taiwan and maintained in conventional animal facilities. BN rats were used as the donor and LEW rats were used as the recipient. Two transplantation models were developed, which included the Osteo-myo-cutaneous (OMC) transplant model and traditional full-limb transplant model. Full-limb model was exactly as the description by Yeh et. Briefly, the limb was cut at the middle of the femur and fixed by intramedullar pin and bone cement, the femoral vessels were anastomosed then sutured the nerves and muscles. Most skin of the limb from donor was saved. The graft of the OMC model consisted all kinds of tissue that full-limb model have which included skin, muscle, nerve and impact bone(tibia). Recipient did not amputate the limb and the graft was suture at the inguinal region. The femoral vessels anastomosed but not repaired the nerves and muscles. Experimental grouping described more detail in table 1. Four immunosupressed protocols were developed which included Leflunomide (LEF) (10mg/kg/day/orally; Hoechst AG, Wiesbaden, Germany) and cyclosporine (CsA) (5mg/kg/day/orally; Sandoz, Switzerland) were given either in short-term (30 days) or long-term (60 days). High dose FK506 (0.5mg/kg/day/IM; donated by Fujisawa, Japan) or low dose FK506 (0.1mg/kg/day/IM) combined with LEF (10mg/kg/day/orally) for 30 days were the other two protocols. Recipients received immunosupressed agents 24hr before transplantation

After stopped giving the immunosupression agents, clinical sign of GVHD or rejection of the grafts were observed. Weight change was gain everyday. The rats in the isograft group were killed at 41 days after transplantation. Other allograft group rats was sacrificed until allograft rejection or the clinical syndrome of GVHD began to improve. Samples were obtains from the following organs of recipient rats: thymus, spleen, mesenteric lymph nodes, esophagus, small intestine, rectum, liver, pancreas, kidney, trachea, lung, skin and bone at necropsy. The sample of skin and bone from the graft were also biopsy. All samples were fixed in 10% buffered formalene for 24hr and embedded in paraffin, cut and stain with hematoxylin-eosin (bone needed decalcium before embedded). Sections were examined by microscope.

The ED1 (Serotec. UK) monoclonal antibodies were used to stain macrophages in the spleen of recipient. Spleen was snap frozen in liquid nitrogen and cryostat sections of 4um were picked up on slides. Cryostat sections were air-dried for 24 hr, fixed in acetone for 10 minutes, 3% H_2O_2 for 30minutes, incubated for 50 minutes in primary antibody, washed and incubated for 30 minutes in mouse anti-rat IgG peroxidase antibody. DAB was used to stain for the peroxidase activity and hematoxline for countor stain. The immunohitochmistry result was analyzed using a computerized digital image analyzed system(Image-pro Plus) on an IBM-compatible PC. Morphometric analyzed of the average area expression of ED1 in 25 random fields(400 X) by brightfield microscope.

Table 1. Experimental groups

Group(n)		Immunosupression	
		Agents**	Time***
1	OMC*(3)	LEF(10)+CsA (5)	30
2	Limb(6)	LEF(10)+CsA (5)	60

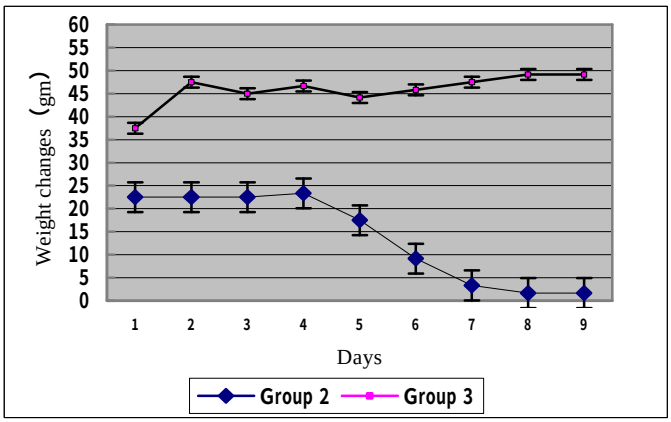
3	OMC(6)	LEF(10)+CsA (5)	60
4	OMC(6)	LEF(10)+CsA (5)	30
5	OMC(3)	LEF(10)+FK(0.5)	30
6	OMC(6)	LEF(10)+FK(0.1)	30

OMC: osteo-myo-cutaneous
*Negative control: LEW-LEW
**mg/kg
***days

Result

Body weight of all animals decreased about one week after transplanting then increased slowly during giving immunosupressant. After stop giving drugs, the body weight curve of limb transplantation group (group 2) became decreasing but other groups were still increasing slowly. (figure 1) In isograft group, no rat showed any evidence of GVHD and rejection when sacrificed. In group 2, two rats of six showed the clinical sign of GVHD which including sudden weight loss, nasal and ocular discharge, poor spirit and dyspnea at 64 and 65 days after transplantation (4 or 5 days after stop giving immunosupresants). The limb graft of other four rats were survival for 71.5 ±2.38 days. OMC grafts of all animals in group 5 was survival for 163± 34.77 days, but did not induce GVHD in any OMC groups. In pathologic examination of the two rats which showing the clinical evidence of GVHD, lung and lymphoid tissues (thymus and mesenteric lymph nodes) were the major target organs. Thymus was largen, congestion and the wall of small intestine became brownish and thin in gross. No findings specific for GVHD could be identified in small intestine and rectum under microscope. Interstitial pneumonitis, leukaemic and macrophagic infiltration around the bronchiole and blood vessel, and intraluminal plugs of degenerate epithelium and mucus in the bronchiole could be found in lung. Thymus was lost the normal structure of the cortex and medulla and many activate macrophage in it. The follicle structure of mesenteric lymph nodes became unclear and the small blood vessels infiltrated in the subcortex. In the OMC model treating with FK506 dose group (group 5, group 6), althought there did not show any clinical sign of GVHD, but the mononuclear cell infiltrating at the tone and bile duck in the liver was noted.

Figure 1.



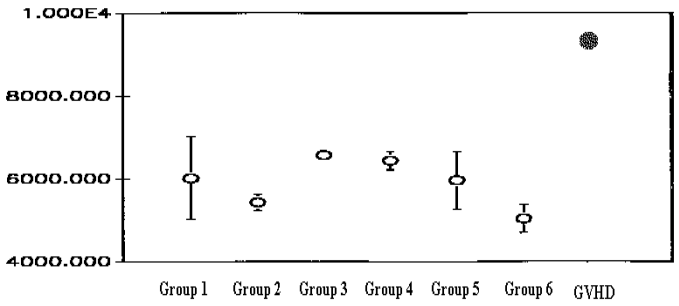
The immunohistochemical stain show that macrophages activity in the spleen of GVHD rats in group 2 was increasing markedly.(figure 2) Other group did not have significant difference ($p>0.01$).

Table 2.

Group(n)		Immunosupression		Clinical observation	Graft survival time	Generral pathology degree
		Agents	Time			
1	OMC *(3)	LEF(10)+CsA(5)	30	NO GVHD NO rejection	41±0	-
2	Limb(6)	LEF(10)+CsA(5)	60	GVHD Rejection	71.5±2.38	++++
3	OMC(6)	LEF(10)+CsA(5)	60	No GVHD Rejection	88.5±4.04	+
4	OMC(6)	LEF(10)+CsA(5)	30	No GVHD Rejection	53.33 ± 14.32	+
5	OMC(3)	LEF(10)+FK(0.5)	30	No GVHD Tolerance	163±34.77	+++
6	OMC(6)	LEF(10)+FK(0.1)	30	No GVHD Rejection	92.17 ± 31.76	++

*Negative control: LEW-LEW isograft

Figure 2.



Discussion

The GVHD after BMT had been subdivide into acute GVHD and chronic GVHD. (1, 7) The clinical incidence of acute GVHD after sibling-matched bone marrow transplantation was 30-60%, which dependent on different protocols. (38, 39)There was still no clinical case of GVHD after limb transplantation today, it developed in 30-50% of recipients animals in experimental model. In our study this time, two of six (33%) show the clinical incidence of GVHD, the incidence was matched to other studies. (3,4,36,37,34) The incidence of both kinds of GVHD was similar to each other, different indeed, the donor and recipient combination in bone marrow transplantation was lower histocompatibilitive (sibling HLA-matched), but in limb transplantation experiments usually used the combination presenting a very strong histocompatibility barrier. Therefore, if lower histocompatibility combination was chosen for limb transplanting, the incidence of GVHD may be much lower. Clinical lesions of skin, diarrhea and liver function disorder were the typical clinical sign of GVHD after bone marrow transplantation and were used to be the criteria for determine clinical degree.(2, 5, 6, 12, 20) Skin, intestines, portal triads in liver, bile duct were the characteristic pathologic target tissues, too.(9,10,40) In our study, the major clinical signs were sudden weight lost and nasal discharge but not the reddish skin or diarrhea. The major histological lesions was in lymphoid tissue and lung but not the skin, intestine and liver. Did Lung lesion due to GVHD or infection had been discuss in some literature. (15, 16, 17, 18) Althought we could not excluding the infection factor, but the lesion lesion like Interstitial pneumonitis, leukaemic and macrophagic infiltration around the bronchiole and blood vessel must correlate to the GVHD. Because, not all rats in the same immunosupression group have the same lung lesion, only these rats have GVHD show the sever typical lung lesion metion in the reference. No matter concerning the clinical signs, incidence, or pathological feature, the GVHD after limb transplantation was different from after BMT. The difference may be due to the following reason, no pre-transplantation regimen (total body irradiation or chemical therapy) needed therefore the tissue of recipient was not been injured. The cytokines release from recipient injure tissue due to pre-transplantation regimen was an important trigger in acute GVHD after BMT.(10,28) Another important factor was that, the recipient in limb transplantation has normal immunological ability which not like bone marrow transplantation.

An appropriate animal model for studying GHVD after limb transplanting is important. The transplanting surgical procedure and the immunosupression protocol are the major factors in it. The limb transplanting animal model in the past studies, which could induce GVHD, usually used all-limb procedure and the immunosupression time was very long from 60 days to 16 weeks. (3,4,22,36,37) Too complicate procedure and long time was not good for a model, therefore we tried the OMC model and short duration immunosupression protocol. As a result, OMC transplanting model could not induce GVHD in clinical as limb model. Although both OMC model and limb model consist the same kinds of tissues, there are still several difference between the two models. First, limb model has more bone marrow than OMC model. The limb graft has all bones below the stifle joint which including the tibia, radius, carpus, metacarpus and phalanges of manus but OMC graft only has tibia. Although VBMT has the microenvirement to support the bone marrow cell to ingraft immediately after transplanting, (35) but the quantity of transplanting cells may still be an very important factor. Secondary, the OMC graft was disparted from the stifle joint. The circulatory system of the tibia may be influent during getting graft

proceeding. Another factor was the nerve, OMC model was not repaired the nerve. Nerve effects on the bone marrow hemopoiesis was not very clear today, the study show that the cell will escape into peripheral blood after injure the nerve, but some study show not.(23,24) At least, the nerve innervation will influence the graft muscle, therefore the muscle of the OMC model was atropy, and the circulatory function must not as good as the limb transplant model.

About the immunosupressant protocols, high dose FK506(0.5mg/kg) combine with leflunomide could provide more protection for graft than cyclosporine. In the group giving high dose of FK506 group (group 5), there was no rats show the clinical evidence of GVHD but some histological lesions the mononuclear cell infiltrating could be found in the liver and tongue, likely which had been found in the GHVD rats in our past study.(21) The mononuclear cell infiltrating is there but not very sever. Therefore if we prolong the time of immunosupression time by high dose FK506, there may be more potency to induce GVHD after limb transplantation.

Macrophage was an important cell in the efferent phase of acute GVHD after BMT because it could release multi-type cytokines like TNF-α, INF-γ.(31, 32) ED1 was a high specific marker for cells of the mononuclear phagocyte lineage and was presented in plasma. The actual activity of this antigen was not clear today but it expression can be correlated to phagocytosis. (29) We found the ED1 expression in the GVHD rats was more than other rats enormously, this may be reflect the activity of macrophage increasing in GVHD duration. Activate macrophage in thymus of GVHD rats could be another evidence to support it.

In conclusion, the GVHD after limb allotransplantation was not completely the same with the GVHD after bone marrow transplantation both clinical sign and pathological feature in our experimental model. An available animal model to induce GVHD after limb allotransplantation must consist more bone marrow cell, less skin and more potential immunosupression protocol after transplantation. The activity of macrophage and NK cell during the GVHD after limb transplantation are similar to the GVHD after bone marrow transplantation.

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