

行政院國家科學委員會專題研究計畫成果報告

放射線應用於肢體移植之研究

Application of irradiation in limb allo-transplantation , a rat model

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一、中文摘要

本實驗利用大鼠的異體後肢原位移植模式，探討以致死劑量的放射線全身照射肢體捐贈者，是否能防止肢體移植中移植物宿主病的發生。

第一組(n=4)中，組織不相容的 BN 大鼠後肢在移植予 LEW 接受者後，於術後第 75±0.289 天被排斥。第二組(n=6)中，以 cyclosporine (CsA) 5 mg/kg/day 與 leflunomide (LEF) 10 mg/kg/day 合併口服投與肢體接受者，自移植前二天開始直至移植後第 60 天，可有效控制排斥反應，肢體平均存活天數為 75.583±3.281 天。停藥後動物(5/5)均自術後 63-65 天出現類似 GVHD 的臨床症狀。第三組(n=11)於移植當天才開始投與 CsA+LEF 至移植後第 60 天，可有效控制排斥反應，肢體平均存活天數為 83.625±5.679 天。停藥後動物(10/11)自術後 63-65 天出現類似 GVHD 的臨床症狀。而有一隻動物(1/11)停藥後並未出現 GVHD 症狀，且出現短暫免疫耐性情形，移植肢體存活至術後 122 天(停藥後第 62 天)。第四組(n=6)的肢體捐贈者(BN)於移植前二天，先接受致死劑量(15Gy)的全身放照射放射線，肢體接受者(LEW)以 CsA+LEF 合併每日口服投與，自移植前二天開始至移植後 60 天，亦可有效控制排斥反應，肢體平均存活天數為 73.167±0.872 天。停藥後三隻動物(3/6)自術後 63-65 天出現類似 GVHD 之症狀。另三隻動物則未出現，直到移植肢體達到排斥終短點。各組間移植肢體感覺與運動功能恢復無異。此結果顯示，以致死劑量放射線全身照射移植捐贈者大鼠，並不能完全避免 GVHD 之發生。本研究之 GVHD，均經組織免疫化學染色確認。

關鍵字：肢體移植、移植物宿主病、全身放射線照射。

Abstract

Following whole limb transplantation from Brown-Norway (BN) to Lewis (LEW) rats, Graft-vs-Host Disease (GVHD) is observed after the administration of immunosuppressive agents is discontinued. In this study, lethal donor irradiation and variation in immunosuppressive schedules were studied in an attempt to prevent GVHD. In group one (n=4, control), all BN limbs were rejected by untreated LEW recipients in 75±0.3 days after transplantation. Combination cyclosporine (5 mg/kg/day) and leflunomide (10 mg/kg/day) were given orally to 3 additional groups of LEW recipients until 60 days following transplantation. In group 2 (n=6), drug treatment began 2 days prior to surgery. Over days 63 to 65, all recipients developed signs of GVHD. The limbs survived for 75.6±3.3 days. In group 3 (n=11), drug treatment began on the day of surgery. All but one rat developed signs of GVHD, comparable to that seen in group 2, over days 63 to 65. The mean limb survival time was 83.6±5.7 days. In group 4 (n=6) donor rats were treated with total body irradiation (15 Gy) 2 days before surgery. Drug treatment of the LEW recipients began 2 days prior to surgery. Three of the 6 rats developed GVHD following discontinuation of drug treatment, and the mean limb survival time was 73.2±0.9 days. The incidence of GVHD was not significantly different (p=0.09, Fisher's exact test) among the 3 treated groups. These results show that neither lethal donor total body irradiation or temporal variation in the administration of

immunosuppressive agents can abolish GVHD in this model of whole limb allotransplantation.

Keywords: limb transplantation, GVHD, total body irradiation

二、緣由與目的

In our previous study, cyclosporine (CsA) and leflunomide (LEF) at the dosage of 5mg/kg/day and 10 mg/kg/day respectively for 60 days successfully prevented limb allograft rejection and with satisfactory functional recovery. However, in this model, potential fatal graft-versus-host disease (GVHD) was universally observed after the discontinuation of immunosuppression(10). GVHD is an immunological conflict manifested by attacking donor immunocompetent cells against alloantigens of the host.

Irradiation of the graft tissues preoperatively has been used in small bowel transplantation (4, 5, 6), pancreas-spleen transplantation (7), and blood transfusion (8) to prevent GVHD. There was one successful attempt in limb transplantation of rat combination as well. The purpose of this study is to evaluate the effect of donor irradiation in preventing GVHD across the highly MHC incompatible BN-LEW barrier.

三、實驗方法

Animals: Male inbred Brown-Norway (BN, RT1ⁿ) and Lewis (LEW, RT1^l) rats were used as allograft donors and recipients, respectively. The combination represents a very strong histocompatibility barrier.

Experimental groups: Group 1 (n=4), BN hindlimbs were transplanted to LEW recipients without immunosuppressive treatment. Group 2 (n=6), LEW received BN allograft and Neoral and LEF of 5 and 10

mg/kg/day from d-2 to d60. Group 3 (n=11), LEW received BN allograft and Neoral and LEF of 5 and 10 mg/kg/day from d-0 to d60. Group 4 (n=6), BN was pretreated by total body irradiation of 15 Gy at d-2. LEW received BN allograft and the same immunosuppression schedule as group 2.

Immunosuppression. Neoral, the microemulsion form of CsA, and LEF were gavaged to LEW recipients at 5 and 10 mg/kg/day, respectively. Neoral solution (100 mg/ml, Sandoz) and LEF powder (Hoechst Co.) were diluted in 1% carboxymethyl cellulose and ultrasonically suspended. The final Neoral and LEF concentration was 5 mg/ml and 10 mg/ml, respectively.

Irradiation. Total body irradiation (TBI) was administered to BN donors at d-2 of 15 Gy. Anesthetized BN lied in the central of irradiation field. Irradiation was provided by Theratronics T1000 Co60, 1.25 MeV source at a rate of 168.5-170.4 cGy/min and at a distance of 100 cm.

Limb allotransplantation. Right hindlimb allotransplantation was performed as previously described (10). The proximal tibial, preoneal, and sural nerve were transected and the muscle were divided at the proximal third portion. Bone fixation was performed with an intramedullary rod and bony cement. After the muscle groups were approximated, vessels and nerves were anastomosed using the microsurgical technique.

Evaluation. LEW recipient were observed daily for general condition, including body weight, and the survival of the grafted limb. If the grafted limb showed rejection signs of erythema, ulceration, and exudation, the animal was euthesized. If the animal showed the clinical signs of GVHD, including weight loss, wasting, diarrhea, hair lost, hunched back, abnormal respiration, and weight loss more than 30%, the animal was euthethized. Three recipients of group 2 showing GVHD

were sacrificed on the day with the most severe clinical signs of GVHD for histopathologic examination. The skin, muscle of the grafted limb and skin, tongue, lymph node, thymus, liver, spleen, and intestine of LEW recipients were sampled for histopathology and immunohistochemical evaluation.

Statistics. The mean allograft survival times of the three groups were compared using Kaplan Miere method of the survival analysis. Animals for GVHD examination were censored.

四、實驗結果

The allografts. A total of 14 rats were operated and treated as described of the 3 groups. All grafted limbs showed mild edema induced by operation for a few days posttransplantation. The control allografts (group1) were rejected in 7.5 days, first from the the skin of the paw and progressed to the whole limb. There was no any signs of function recovery in this group.

The six allografts of group 2, under the cover of CsA and LEF, appeared normal grossly with brown hair regrowing by d13-15 and survived at least 60 days postoperation. Most of them gradually recovered from functional inability since d20, which showed weight bearing, toe spread, and positive sensory stimulous test.

The six allografts of group 3, recieved pre-transplantation irradiation and immunosuppression, encountered the same survival and functional recovery as those in group 2, but the hair did not grow until about d25-30. In addition, the growing hair was still sparse, tiny and white to the end of the experiment.

During the period of immunosuppression, allografts were generally free from signs of rejection. However, subtle edema and erythema appeared in 3 animals, 2 in group2 and 1 in group 3, for a few days and then subsided spontaneously without changing the dosage of immunosuppression and never

happened again until ceasing the drugs. By the end of immunosuppression, the allografts of group 2 and 3 remained intact, in animals died or killed of GVHD, or were rejected. The MST were 7.5 ± 0.3 days in group1, 75.6 ± 3.3 days in group 2, and 73.2 ± 0.9 days in group 3. The MST of group 2 and 3 were stastically significant longer than group 1, and no significant difference between group 2 and 3.

The recipients. All LEW recipients experienced depression and slightly weight loss for a few days immediately after transplantation. The control rats did not show GVHD but only acute rejection and were sacrificed at the rejection endpoint on d7-8. There was no apparent signs of depression of the rats. Their body weight remained the same as it was before transplantation or just declined slightly.

In group 2 and 3, animals showed generally good health conditions manifested with normal activity and steady weight gain in the remaining period of immunosuppression. By d63-65, that was 3-5 days after immunosuppression discontinuation, all rats (n=5) in group 2, excluding the one died of irrelevant cause on d61, showed tachypnea with abdominal respiration, depression, hunch back, anorexia and steep weight loss. Few also showed soft stool and/or diarrhea. Two of the rats returned to normal by themselves on d68, the signs of GVHD disappeared and the body weight went up again. The other 3 for GVHD histology examination were killed with signs of GVHD on D67-68 and one of them seemed to overcome GVHD when sacrificed.

In group 3, three LEW developed signs of GVHD as group 2, and the other 3 did not. Among the three who developed GVHD, one showed severe wasting and died on d70. The other 2 recovered from GVHD by themselves. In the other hand, rats who had no signs of GVHD showed good health condition after ceasing immunosuppression and steady weight gain.

Histopathology. All rejected allografts exhibited classical rejection signs in skin and muscle of the limb microscopically. There were severe diffuse mononuclear cells infiltration, degenerative and necrotic epithelial cells, basal cell vacuolization, bulla formation, dermal edema and mononuclear cell infiltration.

Histologic evidences of GVHD were found in some animals. There were local mild to moderate mononuclear cells infiltration and epidermal cell vacuolization, degeneration in the dermal-epidermal junction of the tongue. Mild mononuclear cells infiltration around the small bile duct. Individual intestine cryptic epithelial cell necrosis, and lymphoid depletion and cystic formation.

Immunohistochemically positive BN cells were stained primarily in pooling lymph nodes, some in mesenteric lymph nodes and spleen, in recipients with signs of GVHD.

五、討論

The clinical signs of GVHD in this study were very consistent. They all appeared on d63-65 and characterized by abnormal respiratory and steep weight loss. But they were different from the classical GVHD signs which characterized by hair loss. The difference may relate to different experiment model. The classical GVHD were often observed in bone marrow transplantation, in which the recipients were irradiated before transplantation. This pretransplantation regimen may result to cytokines such as IL-1 and TNF- α in the skin and so triggered GVHD to present lesions on recipient's skin (1). The other reason may be the different rat strain combination. In the parent to F1 hybrid combination which is completely unbalanced, GVHD is allowed but not allograft rejection. The different immune reaction between strains lead to different presentation, severity and occurrence of GVHD (2, 3).

The object of this study was to prevent GVHD in our established rat limb model by cleaning the immunocompetent cells in grafts

pretransplantation. We use irradiation to do the job and the effect in this preliminary study was not as satisfied as predicted. Only half of the animals receiving irradiation did not show the clinical signs of GVHD. We assumed that the dosage given as 15 Gy TBI was not enough or the way donor receiving irradiation was not proper.

For the dosage of 15 Gy, there may still be some existed radioresistant immunocompetent cells that can triggered GVHD. That also happened in other experiments (9). Clinically, the irradiated limb in group 3 showed abnormal hair growth, so it seemed that increasing the dosage was not relevant.

As for the way irradiation delivered in this study, the anesthetized rats lied on a surface without tissue compensator and rays from the lateral side, may result to an improper dosage that really received by the animal. Because the T1000 irradiator has a high energy source, whose build up region is about 0.5 cm. Thus, the area not as depth as 0.5 cm will receive a dosage less than 15 Gy. And the immunocompetent cells may be alive to initiate GVHD. Consequently, this simple preparation may not suit for our goal in this study. In conclusion, total body irradiation of 15 Gy to the limb donor, although do no harm to the function and the survival of the limb, did not totally abrogate the development of GVHD in the BN \rightarrow LEW limb allotransplantation.

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