Porcine Heterotopic Composite Tissue Allograft Transplantation using A Large Animal Model for Preclinical Studies

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Background: Composite tissue allograft (CTA) transplantation is currently limited by the risks of side effects resulting from long-term high-dose immunosuppression. Therefore, preclinical animal models are essential to help CTA transplantation advance into clinical reality. Evidence has shown that small-animal model (rodents) immunotherapy protocols cannot be directly applied to humans. This study investigated whether a miniature porcine model is reproducible for preclinical studies.

Methods: Based on the concept of vascularized skeletal tissue allograft transplantation, limb heterotopic allograft tissue from a mismatched donor miniature pig consisting of the distal femur, knee joint, tibia, fibula, and surrounding muscle with a vascularized skin paddle model supplied by the superficial femoral vessels was transplanted into recipient pigs. Swine viability and rejection signs of the allograft were monitored postoperatively. Histopathological changes in the allograft tissues were examined using hematoxylin and eosin staining if the allo-skin flap was rejected.

Results: The recipient pigs were ambulatory immediately following surgery. The flaps showed no visible signs of rejection over the first 4 days of observation. The skin flaps appeared bluish-purple and edematous on postoperative days 5~7, and progressed to tissue necrosis and rejection on postoperative days 8~13. Histological examination revealed marked mononuclear cell infiltration and necrotic changes in the all rejected tissues, especial in the allograft skin tissues (skin > muscle > bone > cartilage).

Conclusions: The results showed this the porcine CTA model is reproducible and suitable for preclinical training for human CTA transplantation. Monitoring of the allo-skin flap is a useful strategy to evaluate composite tissue allograft rejection.

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Key words: composite tissue allograft, transplantation, heterotopic.

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Composite tissue allograft (CTA) transplantation has potential applications in the field of microvascular reconstructive surgery.[1] Advances in reconstructive microsurgery, an increased experience with organ transplantation, and recent developments in immunosuppressive therapy have lead to increased interest in CTA research and its clinical applications.[2] CTA transplantation represents an alternative to conventional reconstructive methods for repairing various tissue defects resulting from trauma, surgical resection for tumor extirpation, and congenital defects. This surgical procedure offers patients who lack their own 'autologous' tissue for reconstruction the chance for reconstruction using tissue structurally similar to their own.

The first human hand transplantation was reported from Lyon, France in 1999, where this procedure was demonstrated to be technically feasible.[3] Since that time, multiple hand transplantations have been performed with various levels of success and failure.[4] In spite of its promising potential, CTA transplantation has not been widely adopted for use in clinical settings because of the side effects of long-term immunosuppressive therapy and the phenomena of chronic rejection.[2,5] Therefore, in order to evaluate the long-term efficiency of new immunosuppressive strategies, they must be tested in preclinical trials.

Preclinical animal models are essential to help CTA transplantation become a clinical reality. Investigations involving small-animal hind-limb models are well developed for the evaluation of CTA rejection. Even though the rat model has shown predictable patterns of rejection, differences exists between the human and rat immune systems. Applications of results found in rodent models do not necessarily translate to human studies. Large-animal models are preferable because their immunological responses more closely mimic those observed in humans. Several animal transplant protocols have been used in CTA transplantation including rabbits,[6] dogs,[7] pigs,[8-10] and primates.[11] Large-animal models, especially those using pigs and primates have better characterizations of the major histocompatibility complex (MHC), which is similar to that found in humans. In 1998, Lee et al. described a miniature swine heterotopic limb allograft transplantation model. Results using this model were predictable, and the procedure produced minimal morbidity to the animals.[8] Therefore, in this study, we investigated whether this large-animal model is reproducible so that it can be utilized for our future preclinical studies.

METHODS

Animals

Three transplant donors and 3 recipients were age (3 months old)- and size (15~25 kg)-matched out-bred farm wild-type miniature pigs. Animal care followed guidelines established by the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health, USA. Experiments were conducted under an Institutional Animal Care and Use Committee (IACUC) protocol approved by both the University of Pittsburgh, USA and Chang Gung Memorial Hospital at Kaohsiung, Taiwan.

Transplant procedure in the CTA model

A recipient pig was premedicated with ketamine (10 mg/kg) and xylazine (1.5 mg/kg) through an intramuscular injection. The animal was placed in a supine position on the operating table and intubated. Anesthesia was maintained with an inhalation mixture of 1%~2% isoflurane and oxygen. After normal sterile preparation, the following procedure was performed.

An intravenous catheter was put in place for intraoperative fluid management. This catheter was subsequently used to draw blood samples and administer medicines postoperatively. A single-lumen Hickman catheter was inserted in the internal jugular vein under direct vision and tunneled in a posterior direction to exit high on the dorsal neck. The incisions were closed in layers using both absorbable and non-absorbable sutures.

A composite tissue skeletal graft consisting of the tibia, fibula, knee joint, distal femur, and surrounding muscles was harvested from a donor pig. First, a T-shaped groin incision was made, and the femoral vascular pedicle was isolated. A paddle of skin measuring approximately 8 x 8 cm was preserved on the medial aspect of the knee area, and it was supplied by the superficial femoral vessels. The rest of the skin was removed from the limb. The tibia and fibula were divided at the junction of the upper 1/3 and lower 2/3 (about 5 cm below the knee), and the thigh muscles were divided at the level of the

mid-femur. The femur was divided 5 cm above the knee joint. The graft was perfused with heparinized saline until the recipient site was prepared (Fig. 1A). On division of the vascular pedicle, 60 ml of heparinized saline was flushed through the femoral artery. The donor animal was euthanized with an overdose of pentobarbital once the limb had been harvested.

The recipient animal was prepared in a similar fashion. To decrease the ischemic time during transplantation, 2 surgical teams simultaneously dissected the donor and recipient operative beds. The recipient femoral vessels from the contralateral site relative to the donor pig were isolated by means of a groin incision. A subcutaneous pocket was developed in the anterolateral abdominal wall. The limb graft was placed in the subcutaneous abdominal wall pocket and the vessels were anastomosed end-to-end onto the host femoral vessels with 8-0 Proline and 9-0 Nylon interrupted sutures under microscopic magnification. A defect was made in the host abdominal skin, and the skin paddle on the donor limb was sutured into place. After confirming the vessel patency, the wound was closed in antomical layers with 3-0 Monocryl. The skin was closed with 2-0 Ethilon and stapled autosutures (Fig. 1B).

Postoperative care

Each allograft recipient recovered fully with an uneventful postoperative course (Fig. 1C). The animal was placed in a tubinette vest to protect the indwelling IV catheter. Once the animal had awoken and was breathing comfortably on its own, it was returned to its pen. It was placed in a recovery cage for the first 24 h under close observation. Hematological and chemical indices were evaluated postoperatively by drawing blood. The intravenous catheter was flushed with heparin (2000 U heparin in 1000 ml 0.9% normal saline) twice daily. The animal received a 5-day course of a first-generation cephalosporin and was monitored for signs of distress, sepsis, or wound complications.

Histological examination of tissue sections

At the time of allo-skin rejection, animals were sacrificed; skin, muscle, cartilage, and bone samples were obtained from the donor allograft and fixed in an Accustain® formalin solution (neutral buffered 10% formalin, Sigma, St. Louis, MO, USA). These

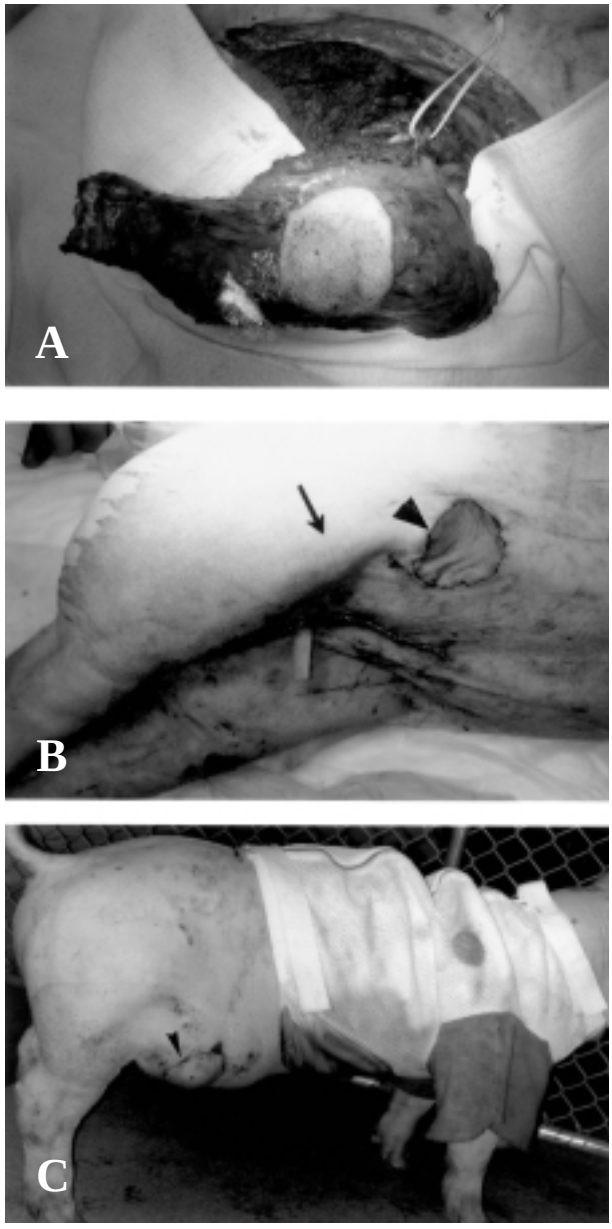

Fig. 1 Heterotopic limb composite tissue allograft (CTA) transplantation model. (A) Intraoperative photo of the harvesting of the left hind-limb CTA including a vascularized skin paddle, skeletal tissue of the femur, tibia-fibula bone, and knee articular joint. A vascularized skin paddle measuring 8 x 8 cm was designed in the medial aspect of the thigh and knee area. The vascular loop identifies the CTA pedicle originating from the femoral vessels. (B) Animal with a heterotopic allograft in place postoperatively (arrow). (C) The skin paddle of the CTA in the recipient's lateral abdominal wall pocket showing no signs of rejection on postoperative day 4.

tissues were then sectioned at 6 μm, stained with hematoxylin and eosin (H & E), and assessed using light microscopy.

RESULTS

The heterotopic allograft transplantation remained viable throughout the first 24 h of monitoring immediately following the return of the animal to the recovery cage. No special care was required for the animals. Following recovery from surgery, the animals ambulated freely in their cages with no difficulty. Postoperatively, the animals almost immediately began to consume food and water. The skin flap was monitored on a daily basis for any signs of rejection including erythema and changes in texture. There were no significant differences in biochemical studies using blood drawn preoperatively and postoperatively. The flap showed no visible signs of rejection over the first 4 days of observation, except that local swelling surrounding the allograft area was noted. The skin flap appeared bluish-purple and edematous on postoperative days 5~7, and tissue necrosis developed on postoperative days 8~13.

However, histological examination following H & E staining revealed marked mononuclear cell infiltration and necrotic changes in the epidermis and dermis of the rejected allo-skin tissue compared to that in the normal control skin (Fig. 2A,E). The muscle and bony tissue showed an inflammatory reaction and mononuclear cell infiltration (Fig. 2F,H). The cartilage of the allograft revealed less mononuclear cell infiltration as compared to other rejected tissue (Fig. 2G). Mononuclear cell infiltration in the rejected allo-skin tissue significantly increased compared to that in the other rejected tissues. These analytical findings indicated that different antigenicities of the composite allografts tissues existed. Allo-skin tissue can be used as a clinical monitor to evaluate whether a CTA is being rejected.

DISCUSSION

Composite tissue allograft (CTA) transplantation offers many advantages over autologous tissue reconstructive procedures including superior functional and esthetic outcomes, no donor site morbidity, and fewer subsequent surgical revisions required.[10] Transplantation of CTAs may provide an attractive strategy for the reconstitution of defects including bone, joints, muscles, or even the peripheral nervous system.[12,13]

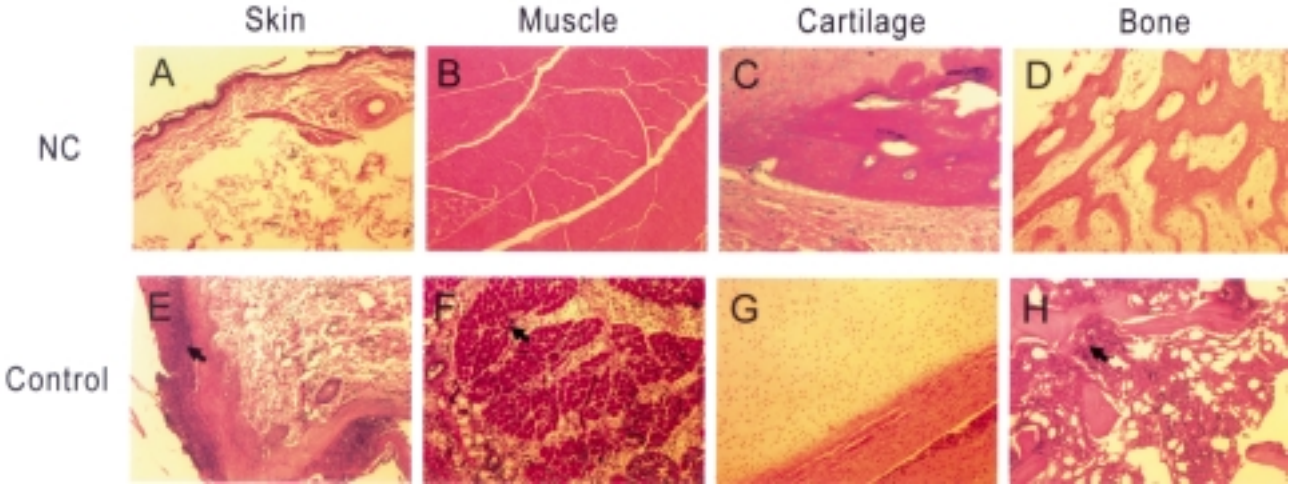

Fig. 2 Histophathologic examination after composite tissue allograft rejection using H & E staining. Normal donor tissues as the normal control (A-D). The rejected tissue of the allo-skin revealed marked mononuclear cell infiltration and necrotic changes (arrowhead) in the dermis and subcutaneous layers as compared to that in normal skin (E). The muscle (F) and bony tissue (H) also showed an inflammatory reaction and greater mononuclear cell infiltration compared to that in normal tissues (arrowhead). The cartilage of the allograft (G) revealed less mononuclear cell infiltration as compared to that of other rejected tissues. Mononuclear cell infiltration in rejected allo-skin tissue was significantly increased compared to that in other tissues. Magnification is 100×.

There have been many reports of whole-limb allotransplantation in experimental rodents.[15-18] However, these models almost all used small animals that are not suitable for applications in humans. Evidence has shown that small-animal model (using rodents) immunosuppression protocols cannot consistently be applied to humans because rodents tend to be more tolerant of allograft transplantation.[19] In order to assess new immunosuppressive protocols and the possibility of tolerance induction, it is essential for large-animal models to be assessed prior to initiation of human clinical trials.

Large-animal studies for CTA transplantation are superior to small-animal models for a variety of reasons. From an immunological viewpoint, the MHC complex in large animals is better characterized, especially in miniature pigs and primates.[20-22] Predictable rejection processes in solid-organ studies that incorporate animals with MHC disparities are similar to those of humans. Pigs have a similar immunological system to that of humans and have extensively been used for transplantation studies.[8,23] Ustuner and Ren described a radial forelimb osteomyocutaneous flap.[10,23] They mentioned that this model is ideal for performing long-term studies investigating allograft survival, and sensory and motor function. However, there are still some difficulties with the ambulatory status of the animal based on the need to put a cast on the allograft.

In this heterotopic CTA transplantation model, the reproducibility of previous allotransplantation results in miniature pigs was explored. The pigs were able to freely ambulate postoperatively. Compared to both heterotopic hind limb and orthotopic forelimb transplantation, this model resulted in minimal morbidity to the animals except for increased abdominal wall girth. However, a disadvantage of this model is that the functional motor and sensory outcomes could not be evaluated following CTA transplantation.

In marked contrast to the monitoring of solid-organ transplants, measurements of graft function cannot easily be utilized as an indicator of allograft rejection. However, since CTAs are not hidden from observation, rejection of the graft might be easily detected and monitored via inspection of the skin. In this study, the heterotopic porcine allograft model allowed simple clinical visualization of the CTA skin surface for detecting early rejection and the vascular status of the allograft.

The study of composite tissue rejection is complex. Different antigenicities of various tissues found within the CTA (skin, muscle, bone, articular cartilage, and blood vessels) result in differential rejection reactions.[14] In this study, histopathological staining was analyzed when allograft rejection occurred. The data revealed that mononuclear cell infiltration in rejected allo-skin was markedly increased compared to that in other rejected tissues. These experimental results indicate that different antigenicities of the various allograft tissues found within the CTA results in various extents of the rejection process. Monitoring and modulation of early rejection of allo-skin may be a key treatment strategy in CTA survival.

In summary, this heterotopic CTA model is reproducible and suitable for preclinical studies in a large-animal model. This procedure caused no major morbidity to the animals while permitting observation of allograft survival. This is an important step towards CTA transplantation becoming available in humans.

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以豬異位複合性組織異體移植做大動物模型以利臨床前研究

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背 景： 複合性異體組織移植目前仍受制於長期高劑量服用免疫抗排斥藥物引發的高危險性副作用。因此，臨床前動物模型研究，以利近一步複合性異體組織移植臨床實際運用有具必要性，證據已顯示使用小動物 (如齧齒類) 免疫治療模式無法直接運用到人體。因此我們嘗試評估用迷你豬複合性組織異位移植模型是否有其重複性，以利未來臨床移植研究。

方 法： 依據帶血管莖骨組織移植觀念，以迷你豬的後肢異位異體複合性組織包括遠端股骨、膝關節、前端脛腓骨、及周邊肌肉組織及股動脈支配之帶血管莖皮瓣複合性組織移植至組織相容抗原不同的受體豬上。移植術中、術後其豬的生命跡象及複合性皮瓣組織移植體皆密切觀察監控中。

結 果： 手術麻醉甦醒後，受贈豬可馬上自行活動。而移植異體皮瓣在術後 4 天並無排斥現象產生。然而在移植術後第 5 至第 6 天左右開始出現局部排斥反應。皮瓣組織有水腫及藍紫色循環不良現象，並逐漸惡化至術後約第 8 至第 13 天組織排斥壞死。組織學切片顯示排斥反應時，此異體複合性移植組織其各種組織皆有顯著單核球浸潤。其中皮膚皮瓣組織尤其明顯 (皮膚＞肌肉＞骨頭＞關節)。

結 論： 此結果顯示以豬作異位異體複合性組織移植模型是可重複製的。其合適於臨床前做爲人體異體複合性組織移植前的訓練模型。觀測皮瓣組織爲一評估組織移植排斥反應有效策略。

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關鍵字： 複合性異體組織，移植手術，異位性。

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