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ORIGINAL ARTICLE

New composite tissue allograft model of vascularized bone marrow transplant: the iliac osteomyocutaneous flap

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Summary

Vascularized bone marrow transplant (VBMT) induces donor-specific chimerism in experimental models across the major histocompatibility barrier. An experimental model for immunotolerance studies should sustain a high antigenicity with low morbidity. Accordingly, we introduced an iliac bone osteomusculocutaneous (IBOMC) transplant model in rat. It consists of a large skin component and an abundant bone marrow cells (BMC) population. We tested this model with isograft transplantations between Lewis rats ($RT1^l$) and with allograft transplantation between Lewis-Brown Norway (LBN $RT1^{l+n}$) donors and Lewis ($RT1^l$) recipients under low dose of cyclosporine A monotherapy. Immunologic responses were tested for donor cell engraftment and chimerism induction. All isografts survived indefinitely and allografts were viable at 200 days post-transplant under low dose of cyclosporine A. Microangiography of the graft revealed preservation of skin, muscle, and bone components. Histologic examination confirmed viability of all allograft components without signs of rejection. Long-term engraftment of donor-origin ($RT1^n$) BMC was confirmed by donor-specific chimerism (1.2%) in peripheral blood and bone marrow (1.65%) compartments and by engraftment into lymphoid organs of recipients. The IBOMC transplant proved to be a reliable composite tissue allotransplantation (CTA) model. Moreover, because of its robust bone marrow component and large skin component, it is applicable to studies on immunologic responses in CTA.

Introduction

Several models of composite tissue allotransplantations (CTAs) contain a bone marrow (BM) component and thus are considered potentially tolerogenic. The BM compartment represents the main source of hematopoietic stem cells, which are pluripotent and capable of differentiating into B and T lymphocytes, neutrophils, monocytes, tissue macrophages, and dendritic cells [1]. These cells may either facilitate graft acceptance or be potentially lethal by inducing a graft-versus-host-disease (GVHD) response [2,3]. One of the goals in CTA is to induce donor chimerism without GVHD. Microchimerism has been established in some solid organ transplants but was not attributed to the success of human allograft transplantation [4–9]. Bone marrow transplantation seems to be a logical approach for chimerism induction specifically, without necessitating recipient conditioning. Transplantation of vascularized BM meets the criteria for chimerism induction without the need for host conditioning. This is possible as in vascularized bone marrow transplantation (VBMT), hematopoietic stem cells are transplanted within their natural microenvironment. This technique does not require BM processing and permits donor cell engraftment.

The commonly used VBMT model is the hind-limb allograft, which is a carrier of BM cells as well as other tissue components including skin, which therefore makes

it an attractive model to test immunologic responses in CTA. But despite these advantages of limb allograft, new VBMT models were developed. An isolated vascularized femur transplantation model was preferred; however, this model does not completely simulate clinical CTAs as it represents only bone graft without other tissue components that are present in limb allograft. VBMT models, without a skin component, were limited to studies on chimerism induction and donor cell engraftment. Thus they do not replicate CTA transplants characterized by a multi-tissue antigenicity. To resolve the dilemma between the immunologic complexity of multi-tissue components of limb allograft and the immunologically less challenging femur allograft models, we introduced a new model of composite iliac bone osteomusculocutaneous (IBOMC) transplant in rat, which simulates clinical CTA transplants resulting from the high antigenicity of a large skin component and vascularized bone component and robust marrow compartment.

Material and methods

Animal care

The research protocol was approved by the Animal Research Committee of The Cleveland Clinic, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. The study was conducted in accordance with the 'Guide for the Care and Use of Laboratory Animals,' published by the National Institutes of Health. Rats were housed in individual cages with controlled 12-h-light-dark cycles, and standard laboratory food and water were available *ad libitum*. All rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). A total of 36 adult Lewis ($RT1^l$) ($n = 28$) and LBN ($RTl1^{l+n}$) ($n = 8$) rats weighing 200–250 g were used in this study. Transplantations were performed under a cocktail of ketamine (30 mg/kg), xylazine (6 mg/kg) and acepromazine (1 mg/kg) anesthesia. Animals were divided into two experimental groups: an anatomic study group and an iliac osteomusculocutaneous transplant group.

Experimental groups

Anatomic study group

Anatomic study was performed to dissect the vessel branches of the main vascular pedicle within the muscle down to the level of muscle perforator to confirm that this perforator supplies skin component of the graft. This assessment also confirmed that the model of composite skin/bone allograft can be based on the same vascular pedicle.

In Lewis ($RT1^l$) rats ($n = 2$), bilateral anatomic dissection was performed to determine vascular anatomy of the iliolumbar artery and vein, the course of vessels through the adjacent muscles, and the number of branches given to the iliac bone and to the overlying skin. A square-shaped skin island, measuring 8×8 cm, was marked over the flank and lateral aspect of the hip of the rat. The skin component was raised as an island flap based on the cutaneous perforator of the iliolumbar vessels. Superior ventral spine of iliac bone was located under the cutaneous perforator. Next iliac bone osteotomy was performed with a chisel, and the entire iliac bone measuring 3×2 cm was attached to the lateral abdominal wall muscle. Then the whole tissue including iliac bone, lateral abdominal muscle and overlying skin was prepared as an island flap. For the pedicle dissection, abdominal wall was opened and all viscera were retracted to follow iliolumbar artery and vein on back muscle to origin on the abdominal aorta and inferior vena cava. Distal end of abdominal aorta and inferior vena cava were ligated and their proximal ends were cut to perform microanastomoses with recipient vessels. Then all components of the IBOMC graft, including the entire iliac bone, adjacent gluteus maximus, iliacus, and lateral abdominal wall muscles, and the overlying skin island, were elevated on the iliolumbar vascular pedicle as a single unit (Figs 1 and 2).

Four IBOMC grafts were elevated on the vascular pedicle in Lewis donors ($RT1^l$) and were transferred to the groin region of Lewis ($RT1^l$) recipients. In two rats, graft pedicles were anastomosed to the femoral vessels while the remaining two grafts ($n = 2$) were transferred to the groin region of Lewis ($RT1^l$) recipients as composite grafts without pedicle anastomosis as a control for non-vascularized graft survival. All animals were observed until day 21 post-transplant.

Next, anatomic dissection was performed in LBN ($RT1^{l+n}$) rats ($n = 2$) each for limb [10], sternum, and vascularized femoral bone models [11] to compare the size of skin components and number of BM cell population in the bone compartment of these models. Following measurements were taken (Fig. 2):

1 Measurements of skin surface area: After dissection, all skin components were harvested from VBMT models, and the total cross-section area of skin was measured using millimetric graph paper (Fig. 2).

2 Evaluation of BM cell population: To compare BM cell populations in different VBMT models, iliac bone, femur, sternum and limb bones were harvested. To simulate the vascularized BM load of the limb transplant, half of the femur and intact tibia (a bone component of limb allograft) were resected from the limb transplant (Fig. 2). BM cells of the iliac bone, femur and sternum were counted using the trypan blue exclusion technique.

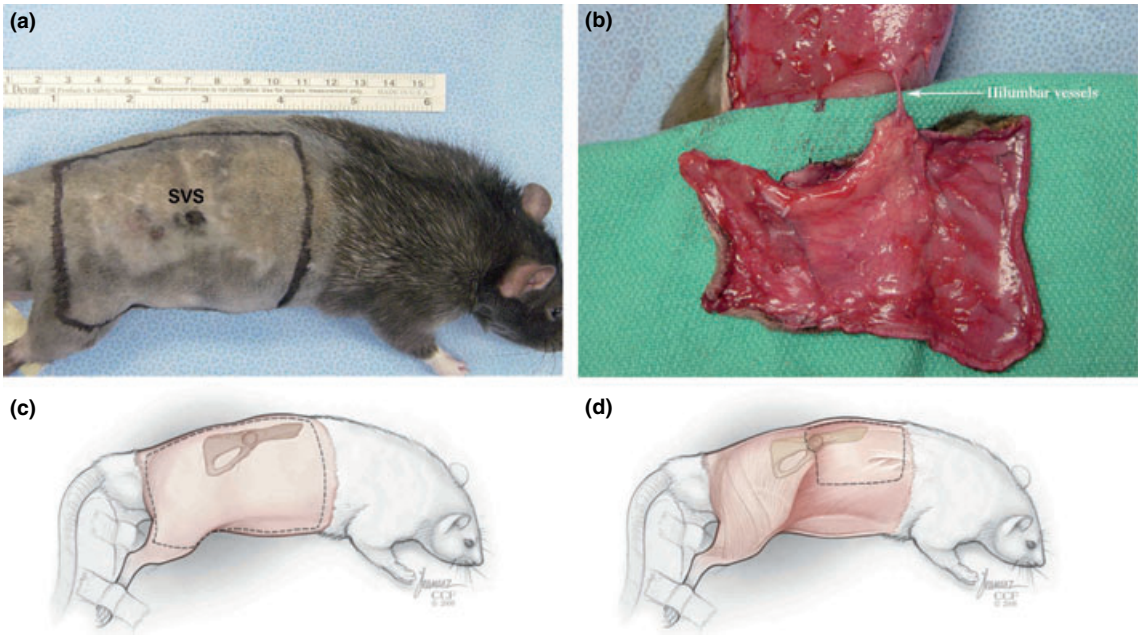

Figure 1 (a) Skin island designed over the lateral aspect of the rat region. (b) Cutaneous perforator of iliolumbar vessels. (c,d) Schematic presentation of skin island and muscle component of iliac bone osteomusculocutaneous (IBOMC) flap.

IBOMC transplant groups

Two subgroups of transplantation were studied. Isograft transplants were performed between Lewis ($RT1^l$) rats ($n = 12$). Iliac osteomusculocutaneous grafts were elevated as described above. In the recipient rat, the left femoral vessels were exposed and dissected up to the inguinal ligament proximally and down to the branching from the superficial epigastric vessels distally. Keeping long pedicle, the femoral artery and vein were ligated and transected before their bifurcation at the popliteal region. The groin skin was resected from the recipient rat to make the defect proportional to the donor flap size. The abdominal aorta and iliolumbar vein of the isograft donor pedicle was anastomosed with the femoral artery and vein of the recipient using the standard end-to-end microsurgical technique with 10-0 nylon sutures (Fig. 2).

After the vessels' patency was confirmed and hemostasis completed, the bone and muscle component of the graft was placed in the inguinal space of the recipient in an oblique position, parallel and inferior to the inguinal ligament. The skin component of the graft was sutured to the edges of the previously created skin defect in the groin region of recipient with absorbable suture material (4/0 Vicryl Ethicon). In the allograft transplant group, iliac osteomusculocutaneous grafts were transplanted from LBN ($RT1^{l+n}$) ($n = 6$) donors to Lewis ($RT1^l$) ($n = 6$) recipients using the same technique described above. Following transplantation, immunosuppression protocol of cyclosporine A monotherapy was used with dosage of 16 mg/kg/day in the 1st week, tapered to 8 mg/kg/day during the 2nd week, to 4 mg/kg/day during the 3rd week and to 2 mg/kg/day during the 4th week and maintained at this level thereafter.

Assessment of transplant viability

Direct observation

Viability of the skin component of isograft and allograft transplants of the IBOMC graft was followed by daily observation. The survival of the skin island was evaluated during postoperative follow up by gross inspection of flap color, edema, erythema, hair loss, desquamation of the overlying skin, bone exposure, and necrosis.

Microangiography

Microangiography was performed to delineate the preservation of vascular territories of the transplant in two animals in the isograft transplant groups on post-transplant day 15. After cannulation of the common carotid artery, 50cc of the mixture (at 70 °C) 50 g of silver oxide, 5 g of gelatin, and 100cc of the 0.9% sodium chloride was injected with a syringe. The grafts were stored at 4 °C for 4 h and underwent radiography with a soft X-ray machine, using Microvision-C mammography film.

India ink injection studies

In two isograft transplants, viability of flaps was evaluated by the presence of dye in the vessels after India ink

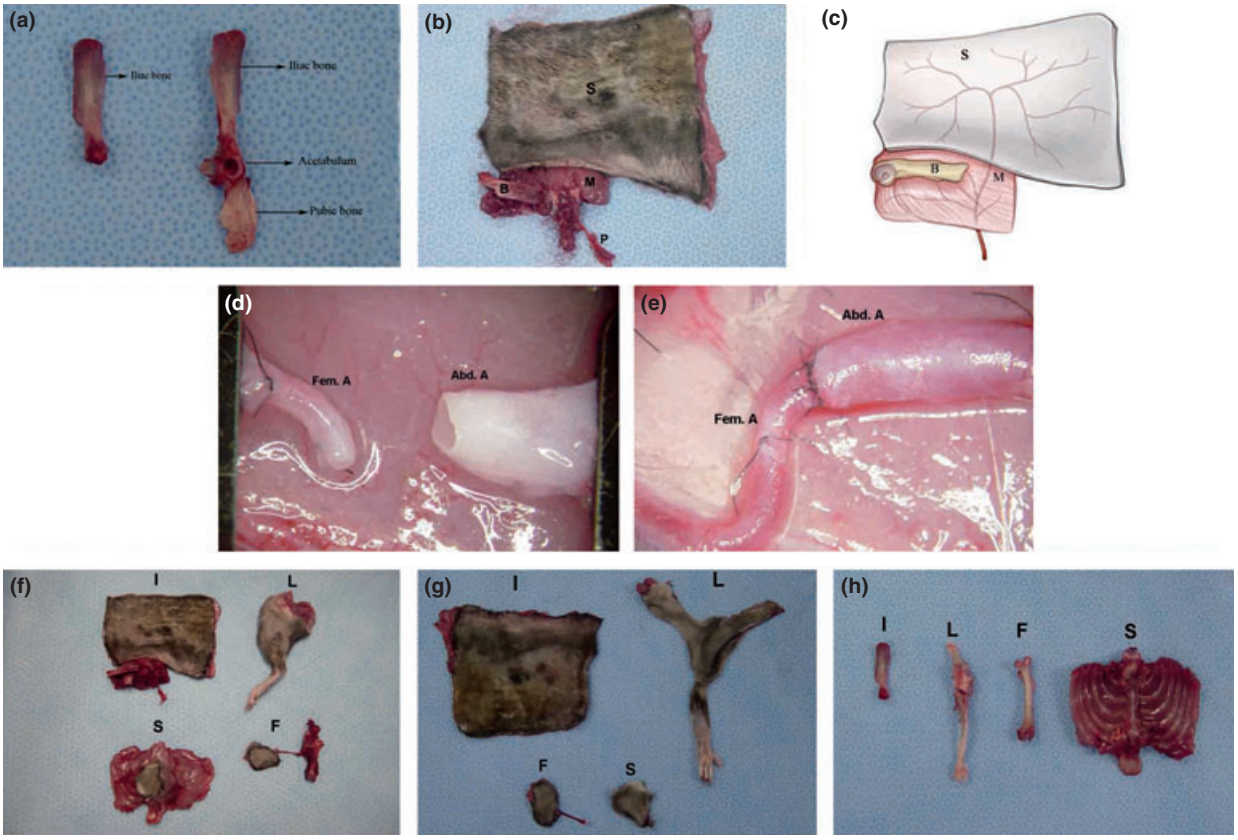

Figure 2 (a) Comparative view of sizes of whole iliac bone and hip bone. (b,c) The composite iliac osteomusculocutaneous flap isolated on the vascular pedicle. (d,e) View of vascular pedicle and recipient's arteries before and after anastomosis. (f) Composition of different vascularized bone marrow transplantation models. (g) Comparision of skin components of vascularized bone transplant models. (h) Harvested bone components of vascularized bone marrow transplant (VBMT) SVS, superior ventral spine of iliac bone. Ib, iliac bone; Hb, hip bone; S, skin component; M, muscle component; B, bone component; P, vascular pedicle; Fem. A, femoral artery; Abd. A, abdominal aorta; I, iliac osteomusculocutaneous allograft model; L, limb allograft model; S, sternum allograft model; F, femur allograft model.

injection at post-transplant day 15. Following cannulation of the common carotid artery with a 24-gage catheter, 5 ml of India ink was injected. The graft was harvested and the bone segment was decalcified using commercial decalcifying solution (Protocol decalcifier A; Fischer Scientific, Pittsburgh, PA, USA) for 3 days. All graft components were stained with hematoxylin and eosin (H&E), and examined under light microscopy for the presence of India ink in blood vessels of the skin, muscle and in the BM and cortex of iliac bone.

Histology

On post-transplant day 200, animals were euthanized; isograft ($n = 2$) and allografts ($n = 6$) were harvested; skin and muscle components were stored in 10% buffered formalin; and paraffin sections were stained with H&E for evaluation. The bone components were decalcified and sections from the iliac bone were processed and stained routinely. Each slide was evaluated by a pathologist blinded to the study for viability of skin, muscle and bone; for the presence of hematopoietic cells; and for signs of fibrosis and rejection.

Immunologic evaluation

Flow cytometry analysis

Flow cytometry (FC) established the presence of donor-specific chimerism for donor MHC class I ($RT1^n$) antigen in the peripheral blood of Lewis recipients during observation time at 7, 21, 63, 100 and 150 days post-transplant. Chimerism was assessed by a combination of conjugated mouse anti-rat mAb $RT1^{n\text{-}FITC}$ (for MHC class I of donor cells $RT1^n$, clone MCA156; Serotec Ltd, Oxford, UK) with mAb specific for T cells: $CD4^{-PE}$ (clone OX-35), $CD8a^{-PE}$ (clone OX-8), B-cell $CD45RA^{-PE}$ (clone OX-33) and monocytes, granulocytes $CD11_{b/c}{}^{-PE}$ (clone OX-42) (BD Bioscience Pharmingen Inc., San Diego, CA, USA). After incubation, samples were lysed and fixed with 1% PFA

solution. Negative control panels were tested and included isotype-matched antibodies (IgG_1^{-FITC}/IgG_2^{-PE}) and phosphate-buffered saline samples. Analysis was performed on 1×10^4 cells using FACS SCAN (BD Bioscience Pharmingen Inc., San Diego, CA, USA) and CELL QUEST software.

To evaluate donor cell engraftment into Lewis ($RT1^l$) BM compartment, BM samples were harvested from the recipient contralateral bone by aspiration biopsy and evaluated by flow cytometry using mouse anti-rat mAb $RT1^{n\text{-}FITC}$ (for donor MHC class I in combination with mouse anti-rat mAb $CD90^{-PE}$ (BD Bioscience Pharmingen Inc.) as described above.

Immunohistochemical analysis

The migratory potential of donor-specific cells into lymphoid organs of IBOMC allograft recipients was evaluated using mAb specific for donor MHC class I. Lymph nodes, spleen, and thymus were harvested from the euthanized representative rats at day 200 post-transplant. Samples were immediately snap-frozen in liquid nitrogen, cut (4 μm), and fixed for 10 min in acetone, air-dried, and blocked for endogenous peroxidase before incubation with mouse anti-rat $RT1^n$ (for MHC class I) mAb for 30 min at room temperature. The binding of primary antibodies was detected using a DAKO LSAB2 System (DAKO, Carpinteria, CA, USA), peroxidase, and aminoethylcarbazole (AEC) in accordance with the manufacturer's instructions. Slides were counterstained in hematoxylin and mounted in Faramount. Sections incubated with nonspecific IgG were used as negative control.

Results

Anatomic dissection studies established a repeatable anatomic pattern in all operated animals and revealed that the iliolumbar artery, which was found to supply the IBOMC graft, originated from the abdominal aorta caudal to the origin of the renal artery, whereas the iliolumbar vein originated from the inferior vena cava at approximately the same level. The iliolumbar artery branched into the lumbar and iliac branches, and during its course a small arterial branch directly supplied bone which branched off before leaving the abdominal cavity at the level of the upper and lateral border of the iliac bone. This branch, penetrating the skin, supplied the flank and hip regions.

In the anatomic study group, at day 21 post-transplant, large composite grafts without vascular supply became totally necrotic whereas, as expected, all skin islands of the IBOMC graft were viable. Histologic examination of the skeletal muscle and bone segments showed skeletal muscle cells, which appeared normal and viable osteocytes within the bony trabeculae. However, examination of the graft group without vascular supply revealed nonviable, necrotic skeletal muscle cells and lacunae lacking osteocytes (Fig. 3).

Comparison of the IBOMC transplant with other VBMT models is presented in Table 1. When weights of CTA/VBMT models were compared, limb transplant was found to be the heaviest graft (18.84 g), followed by iliac (15.70 g), sternum (14.80 g), and vascularized femoral bone (2.4 g) graft. Measurements of the skin island surface area based on the diameter of different VBMT models revealed the following surface area values: IBOMC, 62.96 cm^2; limb transplant, 24.12 cm^2; sternum, 5.4 cm^2; and skin component of vascularized femoral bone, 5.04 cm^2.

The count of BM cell numbers in VBMT models revealed the following values from highest to lowest cell counts: vascularized femur, 50×10^6; limb (including tibia and a half femur), 48.75×10^6; iliac bone, 25×10^6; and sternum, 7.5×10^6 (Table 1).

Clinical assessment of IBOMC transplants

Direct observation

During follow-up period up to 200 days post-transplant, skin islands of all flaps in isograft ($n = 2$) and allograft ($n = 6$) transplant groups were completely viable (Fig. 4a).

Microangiography

Microangiograpic study revealed preserved vascular territories of all components of the grafts, including skin, muscle, and bone compartment (Fig. 4b).

India ink injection studies

Dye study with India ink demonstrated ink uptake by the vessels in the BM and cortical bone, confirming bone perfusion via the vascular pedicle (Fig. 4c).

Histologic examination

Day 200 post-transplant, histologic examination of IBOMC allotransplants did not reveal any signs of rejection, such as blood cell extravasation, edema, necrosis or pigment incontinence. The normal architecture of the skin, skeletal muscle and bone was preserved, and the skeletal muscle cells and osteocytes within the bone trabeculae were viable (Fig. 4d).

Immunologic evaluation

Donor chimerism was evaluated as a sum of donor-origin T-lymphocytes ($RT1^n$/CD4, $RT1^n$/CD8), B-lymphocytes ($RT1^n$/CD45RA) and granulocytes/monocytes ($RT1^n$/$CD11_{b/c}$). The kinetics of chimerism is represented as the

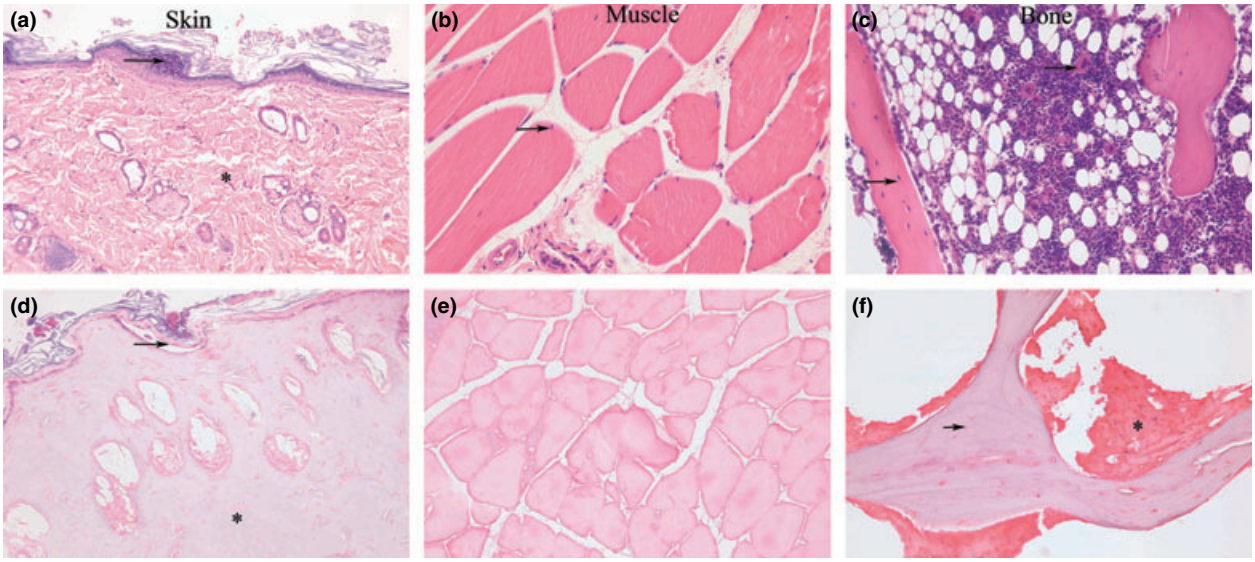

Figure 3 Histologic sections of isograft Lewis (RT1^{l}) demonstrate viability of transplant components at day 21 post-transplant. (a) Intact epidermis (black arrow) and dermis (*) (hematoxylin-eosin stain; original magnification ×50). (b) Viable myocytes in the skeletal muscle components. Multiple nuclei (black arrow) lie at the periphery of the muscle fibers (hematoxylin-eosin stain; original magnification ×400). (c) Viable bone trabeculae and bone marrow components. Osteocytes in lacunae (black arrow), hematopoietic cells (*), and megakaryocytes (white arrow) (hematoxylin-eosin stain; original magnification ×200). Histologic sections of composite isografts [Lewis (RT1^{l})] without vascular supply show necrotic and disturbed components at postoperative day 21. (d) Diffuse necrosis of the epidermis (black arrow) and dermis (*) (hematoxylin-eosin stain; original magnification ×200). (e) Nonviable, necrotic skeletal muscle cells without nuclei (hematoxylin-eosin stain; original magnification ×200). (f) Necrotic bone segment with empty lacunae (black arrow) and a disturbed and necrotic bone marrow component lacking hematopoietic cells (*) (hematoxylin-eosin stain; original magnification ×400).

Table 1. Comparison of iliac, limb, femur, and sternum VBMT models for size of skin component, weight of the graft, and number of bone marrow cell populations within the bone compartment.

VBMT models	Skin surface area (cm^2)	Weight (g)	Bone marrow cell populations (10^6)
Iliac	62.96	15.70	25
Limb	24.12	18.84	48.75
Femur	5.4	14.80	50
Sternum	5.04	2.4	7.5

VBMT, vascularized bone marrow transplant.

total chimerism level of donor-origin cells (RT1^{n}) in the peripheral blood and BM compartment of recipients.

Donor-specific chimerism in peripheral blood of IBOMC allograft recipients was assessed below 1% up to 100 days post-transplant, and at 1.2% at 150 days post-transplant.

Donor chimerism in the BM compartment of IBOMC allograft recipients was assessed below 1% at 100 days post-transplant. By contrast, at day 150 post-transplant, higher chimerism was confirmed by the presence of 1.65% of donor-origin RT1^{n} cells in the recipient BM compartment. Comparison of donor-derived chimerism in BM and peripheral blood of recipients is illustrated in Fig. 5a.

Donor-specific chimerism in the lymphoid tissues of recipient

Engraftment of donor-origin cells (RT1^{n}) at day 200 post-transplant was confirmed in the spleen and lymph nodes but not in the thymus of iliac osteomusculocutaneous allograft recipients. In the spleen numerous donor-derived cells were localized in the white pulp, whereas in the lymph nodes donor-derived cells were confined to the germinal center of the lymph node follicle (Fig. 5b).

Discussion

In the search for minimal immunosuppressive and tolerance-inducing protocols, researchers evaluate the role of donor chimerism in CTA acceptance and survival [12–14]. The role of chimerism in prolonging the survival of organ allografts has been documented [15–17], but there are also reports showing a lack of correlation between chimerism and tolerance [8,18]. Studies designed to ascertain a correlation between the establishment of chimerism, and donor-specific tolerance, are of special

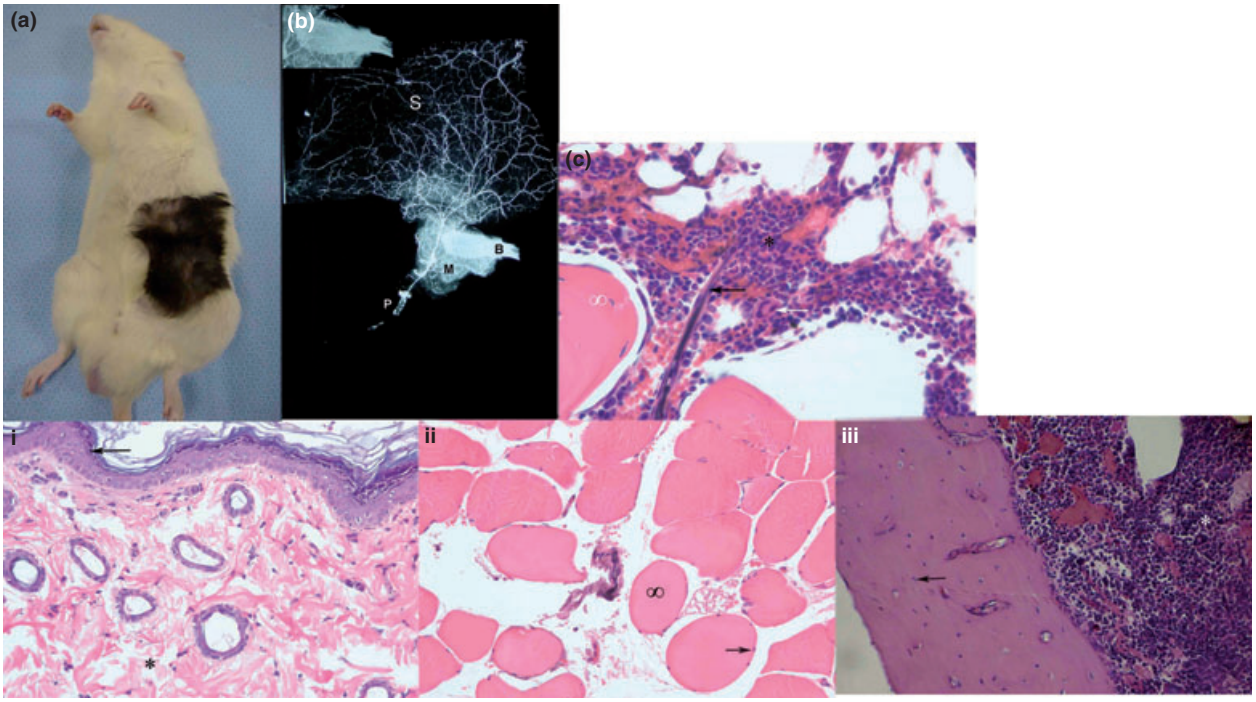

Figure 4 (a) Assessment viabilities of recipients of iliac bone osteomusculocutaneous (IBOMC) allograft Lewis ($RT1^{l}$) from LBN (RTl^{1+n}) donor at post-transplant day 150. (b) Microangiographic analysis of isograft transplant demonstrates the course of the pedicle. The vascular filing with contrast material is depicted in the bone (small picture at the corner of frame). S, skin; M, muscle; B, bone; P, pedicle. (c) Histologic examination of the bone marrow and bone trabeculae from the isograft transplant demonstrates perfusion of the bony component, evidenced by the presence of ink in vessels (black arrow) at post-transplant day 15. Hematopoietic cells (*) and megakaryocytes (white arrow) within the bone marrow and osteocytes within the bone trabeculae (∞) (hematoxylin-eosin stain; original magnification ×400). (d) Histologic sections of vascularized isograft [LBN ($RT1^{l+n}$)] demonstrate viability of the transplant components at post-transplant day 200. (i): normal epidermis (black arrow) and dermis (*) (hematoxylin-eosin stain; original magnification ×50). (ii): nuclei (black arrow) and healthy fibers (∞) in intact skeletal muscle cells (hematoxylin-eosin stain; original magnification ×400). (iii): viable osteocytes within lacunae (black arrow) and hematopoietic cells (*) within the bone marrow. (hematoxylin-eosin stain; original magnification ×200).

interest in CTA transplantation [19], as many experimental CTA models contain a viable donor BM compartment, which may facilitate the induction of chimerism [20–23]. This unique feature of the BM component of CTA has the potential to facilitate hematopoietic stem cell engraftment and repopulation, resulting in chimerism maintenance via the self-renewal of BM cells [24–26].

The VBMT model offers other advantages; specifically, the maintenance of the proliferative capacity of donor BM cells within its own environment, as well a continuous seeding capacity of donor BM cells for homing to different recipient compartments. Moreover, it is believed that VBMT provides a continuous supply of donor-derived progenitor cells having the ability to reconstitute recipients faster compared with an equivalent volume of isolated BM cells [27]. This ability stems from the fact that stromal cells provide mechanical support for hematopoietic cells, as well as humoral factors and growth factors sequestered within extracellular matrix molecules [28,29]. The effect of VBMT transplantation for chimerism induction and maintenance was confirmed in our recent studies where transplantation of vascularized bone containing BM cells resulted in efficient engraftment of donor-origin progenitors into BM compartment and multiple hematolymphoid organs of VBMT recipients [26].

It is known that in BM cell infusion, where the aim is to replace the host's defective lymphohematopoietic system by donor's hematopoietic cells, early and complete chimerism carries the significant risk of inducing GVHD [30]. In contrast, long-term VBMT recipients, with or without irradiation, remain healthy and demonstrate chimerism without clinical or histologic signs of GVHD [31]. Thus VBMT with intact marrow cells, including progenitor cells, and a stromal supporting environment, is superior to conventional cellular marrow infusion [19].

The example of VBMT is rat limb transplant model–the first and standard CTA model [10]. Since this first report, it has been demonstrated that a microsurgically transplanted hind-limb allograft contains donor BM, which is considered as an immunomodulatory factor

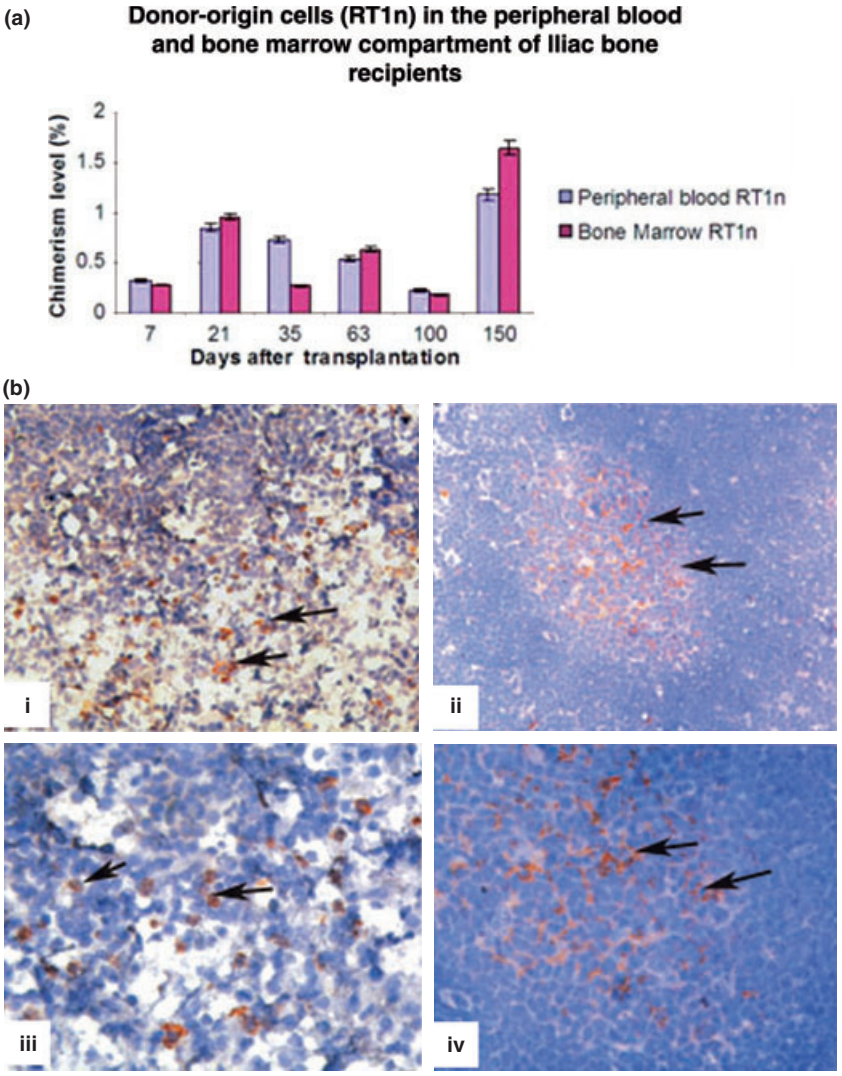

Figure 5 (a): Kinetics of total chimerism in the peripheral blood and bone marrow compartment of iliac bone recipients. Chimerism is presented as a mean (± SD) value of donor-origin cells determined by monoclonal antibody specific for MHC class I antigens ($RT1^n$). (b) Donor-origin cells ($RT1^n$) in the spleen and lymph node of iliac bone representative Lewis recipient ($RT1^1$) at day 200 post-transplant. In the spleen donor, origin cells ($RT1^n$) were localized in the white pulp region. In the lymph node, the presence of donor-derived cells was restricted to the germinal center of the lymph node follicle [immunoperoxidase staining, aminoethylcarbazole (AEC), magnifications: i, ii ×200; iii, iv ×400].

facilitating allograft survival [20,32]. The limb allograft also contains lymph nodes, neurovascular structures, muscles, cartilage, tendon, ligaments, and most importantly the skin, which is regarded as the most immunogenic tissue of the body [33]. The use of VBMTs for inducing chimerism and tolerance gained popularity after limb allograft transplantations were performed successfully [21,32]. However, hind-limb transplantation is technically complex and leaves the recipient with a functional deformity. In this complex model, half of the femur and the entire tibia from the donor are the sources for VBMT. Some investigators have reported major complications such as respiratory failure, bleeding, and thrombus formation following limb transplants. As a result, published mortality rates have been in the range of 20%-30% [34,35]. Thus, because of technical challenges and high morbidity reported in the hind limb allotransplant model, recent studies have focused on new vascularized BM sources. A vascularized sternum model is described as technically simple [36]; however, the transplant procedure can be challenging because intra-abdominal vessels must be used in recipients. Furthermore, intra-abdominal placement of VBMT makes follow-up monitoring of the graft impossible. In anatomic study group, we have shown that the sternal transplant model including skin island can be compared with other CTA models containing skin/bone component. However, the sternal allograft was found to have the lowest number of BM cells compared with other VBMT models.

The femur is another source of VBMT. Although there are only few reports on isolated femur allotransplantations [26,37–40], researchers are familiar with this model as, in limb allograft, the femur is considered as a source of vascularized BM cells [20,23,41]. In this model, no

nerves, muscles, or bones of the recipient are removed, and after transplantation the recipient can use the limb without restriction. Disadvantages of this model include inability to monitor graft viability, as the allograft is buried under the skin; and in contrast to the limb transplant, fewer BM cells are available. In contrast to the limb transplant, vascularized femur transplant does not carry highly immunogenic skin-specific antigens thus allows to detect only immunologic effects of VBMT [26,40,41]. For this reason, we modified this model to increase tissue-specific antigenicity and to permit monitoring of the vascularized femur viability by inclusion of the vascularized groin flap. The skin island of the composite femoral bone allograft model is usually small; therefore the skin-related immunologic responses may be less pronounced as compared with that of the limb transplant.

Thus the best VBMT model should closely simulate CTA transplants and carry the immunogenicity of skin and BM component. Accordingly, to meet these needs, we introduced in our study a new IBOMC allograft model that has all the required features including presence of intact iliac bone containing BM cells, which is a prerequisite for VBMT models and presence of the skin component. We expected that in large surface area of the skin component of IBOMC transplant, we would be observing higher level of donor chimerism. This hypothesis was not confirmed, and in IBOMC model donor chimerism was low when compared with limb allograft model. This may be explained by the CsA effect on bone marrow cells. It is known that CsA did not affect hematopoiesis; however, low dose of CsA had an antiproliferative effect on hematopoietic and lymphoblastoid cell lines, and had limited stimulatory effect on CFU-GM [42,43]. In our model, low dose of maintenance therapy with CsA could delay repopulation of donor-origin cells in the recipient bone marrow compartment and this may explain delayed occurrence of donor-origin cells in the periphery. Moreover, this phenomenon may be explained by anatomic differences between IBOMC and limb model where, in contrast to IBOMC, limb contains lymph nodes as an integral part of the graft. The presence of lymph nodes within the graft may contribute to high level of chimerism as observed in our face allograft model [44]. Face transplants represent an example of vascularized skin allograft (VSA) model. However, in contrast to other VSAs such as groin flap or total abdominal wall (TAW) containing only skin and subcutaneous tissues, face allograft includes numerous lymph nodes of the neck and face region which are permissive for chimerism induction. Our recent studies assessing the role of different sizes of skin allografts on chimerism induction, confirmed presence of low chimerism in groin VSA and TAW transplants [45,46], whereas high donor chimerism was observed only in face allografts containing lymph nodes as a component of CTA [44].

Another important component of CTA, which significantly contributes to chimerism development, is bone with the BM cells. Our recent studies of isolated VBMT model without skin component confirmed low level of donor-specific chimerism in the peripheral blood of long-term surviving recipients, similar to the level observed in IBOMC recipients [26]. Moreover, in limb allograft and VBMT models, we have observed engraftment of donor-origin cells into spleen, lymph nodes and thymus [26,47]. In contrast, in IBOMC, face allograft and VSA models engraftment of donor-origin cells was confirmed in the spleen and lymph nodes but not in thymus of recipients [44]. This may be explained by the different immunosuppressive protocols used in these studies. Limb allograft and VBMT were performed under 7-day immunodepletive protocol of anti αβ-T-cell receptor mAb and CsA, whereas IBOMC, face allograft, TAW and VSA were treated with low-maintenance nontoxic dose of CsA monotherapy. These observations suggest that short-term immunodepletive protocol facilitates development of intrathymic microchimerism, which may be permissive for tolerance induction [47]. The paucity of donor-origin cells in the thymus of CsA treated recipients may reflect CsA-mediated lymphokine downregulation and disruption of thymic function, which is essential for cell-homing and engraftment. CsA therapy induces changes within the thymic microenvironment leading to reduction of the size of thymic medulla, decreasing number of interdigitating cells and changing morphology of the epithelial cells [48]. All these changes limit donor cell engraftment and thymic chimerism development. However, low-dose CsA maintenance protocol, used in this study, is permissive for 'prope' tolerance induction [49] resulting from pharmacologic action of CsA, which alters immune response by inhibiting maturation process of dendritic cells and IL-2 production by T cells [50].

These findings confirm that the outcomes of VBMT relate to multiple factors including specific responses of different tissue types, size of the skin component of CTA, presence of pluripotent BM cells and type of immunosuppressive protocols.

Conclusions

This study confirms that IBOMC is a reliable and technically feasible new CTA model to study immunologic responses of different tissue components including vascularized bone with pluripotent BM cells making it an attractive addition to the armamentarium of VBMT models.

Authorship

SN: participated in research design, participated in the performance of research, participated in writing the paper and participated in the data analysis. AK: participated in the performance of the research, participated in writing the paper and participated in the data analysis. ES: participated in the performance of research. MB: participated in the performance of research. SG: participated in the performance of research. MS: participated in research design, participated in writing the paper and participated in the data analysis.

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