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Plasma microRNAs Are Potential Biomarkers of Acute Rejection After Hindlimb Transplantation in Rats

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Background. The development of effective immunosuppressive regimens has resulted in many cases of successful hand transplantation. Visual skin inspection and histological evaluation are used to assess the rejection of hand transplants, but these methods are largely subjective. In this study, we aimed to determine the potential of microRNAs (miRNAs) as biomarkers for acute rejection in vascularized composite allotransplants. **Methods.** In allograft group, 7 male Brown-Norway rats (RT1^n) were used as donors and 13 male Lewis rats (RT1^l) were used as recipients. In control group, 8 Lewis rats were used as donors and recipients. The hindlimbs of donor rats were transplanted orthotopically to recipient rats. Skin changes were noted daily. Skin biopsies were obtained from 5 recipients and evaluated histologically. Plasma samples were obtained from the other 8 recipients before transplant and 7, 10, and 14 days posttransplant and used to measure miRNA expression. **Results.** Skin changes occurred at a mean of 11.0 days posttransplant. Rejection in most skin biopsies taken 7 and 10 days posttransplant was histologically classified as grade 0, whereas that in most biopsies taken 14 days posttransplant was classified as grade 3. We found that expression of miRNA-146a and miRNA-155 was significantly upregulated at 10 and 14 days posttransplant compared with that at 7 days posttransplant. In control group, there were no significant changes in plasma miRNAs expressions. **Conclusions.** The upregulation of plasma miRNA-146a and miRNA-155 was detected before the histological evaluation methods could diagnose complete rejection in the rat hindlimb transplantation model. Plasma miRNA-146a and miRNA-155 may be potential biomarkers of acute rejection after vascularized composite allotransplantation.

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Limb transplantation is considered to be a treatment option for amputation resulting from surgical excision of tumors, accidents, or congenital malformation.[1,2] Transplantation of a human hand was attempted in 1963 in Ecuador, but it was rejected after 2 to 3 weeks.[3] Thanks to the development of effective immunosuppressive regimens in the 1980s, the first successful human hand transplant was performed in France in 1998.[4] However, this graft was removed after 29 months because of the recipient's drug noncompliance.[5] The second successful hand transplant was performed in 1999 and is the longest surviving case in the world.[6] Subsequently, many hand and forearm transplants have been successfully performed and have achieved substantial functional and aesthetic results.[1]

To overcome acute and chronic rejection of transplanted limbs, long-term, and high-dose immunosuppression is necessary, which causes serious side effects including opportunistic infection, malignancy, and organ damage.[7,8] These side effects may be occasionally life threatening. Early recognition of acute rejection allows appropriate control and adjustment of the immunosuppression. Visual skin inspection and histological study are used to assess acute rejection in hand transplants, but these assessment methods are largely subjective.

MicroRNAs (miRNAs) are single-stranded noncoding RNAs 19-25 nucleotides in length. They exist in various tissues, organs, and even in blood, and persist stably.[9] miRNAs have a biding site in the 3′ or 5′ untranslated region of the target messenger RNA and regulate translational repression of protein or degradation of messenger RNA.[10-13] Recently, a number of miRNAs are found to be involved in the innate immune systems and the activation and regulation of inflammatory response.[10,14] Indeed, some studies reported that expression of miRNAs was markedly enhanced or suppressed in several diseases including infection, organ cancer, or autoimmune diseases, and they are considered to be candidate noninvasive biomarkers.[10,11] In addition, some reports revealed that plasma expression of miRNAs was changed in acute rejection after organ transplantation, including heart or kidney transplantation.[15-17] However, there are no reports of plasma miRNA expression changes in acute rejection after limb transplantation. In this study, we aimed to investigate the profile of microRNAs in acute rejection of vascularized composite tissue transplants in rats.

MATERIALS AND METHODS

Animals

In allograft group, 7 male Brown-Norway rats weighing 210 to 290 g (major histocompatibility type RT1^n), were used as the donors. Thirteen male Lewis rats weighing 230 to 330 g (major histocompatibility type RT1^l) were used as the recipients. Of 13 recipients, 5 were used for tissue biopsies, and the remaining 8 were used for visual skin inspection and blood sampling. In control group, 4 and 7 Lewis rats weighing 220 to 320 g were used as donors and recipients, respectively. Of 7 recipients in the control group, 2 were used for tissue biopsies, and the remaining 5 were used for blood sampling. All animals were housed in a room with a daily 14-hour light/10-hour dark cycle with free access to food and water. This research was approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, and all procedures described below were performed in accordance with the Guidelines of the Animal Research Committee.

Surgery

Before surgery, the rats were deeply anesthetized with sodium pentobarbital (40-50 mg/kg) intraperitoneally. Both hindlimbs of the donor rat were perfused with cold heparinized lactated Ringer solution, then amputated at mid-thigh and kept in cold heparinized lactated Ringer solution until transplantation. Each harvested hindlimb was transplanted orthotopically to separate recipient rats. An 18G needle was used as the intramedullary fixation for the femur. The dorsal side muscle and skin were approximated with a 4-0 nylon suture. The femoral vein and artery were anastomosed with a 10-0 nylon suture using an operating microscope. Anastomosis of the femoral vein preceded that of the femoral artery. After ascertaining the patency of the anastomosed vessels, the ventral muscle was anastomosed and the skin was closed with a 4-0 nylon suture.[18] We administered 1 mg/kg per day FK506 by intramuscular injection to the recipient rats for 7 days from the day of the surgery.[19]

Visual Skin Inspection

For 8 of the 13 recipients, the skin appearance was monitored daily until the 14 days posttransplant and assessed for swelling, erythema, keratolysis, and hair shedding.

Plasma Sampling and microRNA Measurement

Plasma samples were obtained from the same 8 recipients used for visual skin inspection in the allograft group and 5 recipients in the control group. We did not sample blood from the 5 recipients used for tissue sampling because the invasive skin biopsies might have affected plasma miRNAs expression. Plasma samples were taken at 1 point during 1 to 3 days before transplant and at 7, 10, and 14 days posttransplant. Approximately 250-µL blood was obtained at each time point from each recipient's caudal vein and the plasma was separated. Total RNA was isolated from approximately 100-µL plasma using the mirVana Paris kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Several miRNAs were reverse transcribed to cDNA using a Taqman miRNA reverse transcription kit (Applied Biosystems) and measured and analyzed by real-time quantitative polymerase chain reaction (RT-qPCR) using the 7500 real-time PCR system (Applied Biosystems). The RT-qPCR data for the miRNAs were analyzed by delta-delta Ct analysis using synthetic ath-miR159a (UUUGGAUUGAAGGGAAGCUCUA) as a standard for miRNA. The amount of each miRNA relative to ath-miR159a was determined.[3,15,20,21]

Tissue Sampling

Skin biopsies were obtained from the transplanted hindlimb of the remaining 5 recipient rats in the allograft group each day from 7 to 14 days posttransplant and the remaining 2 recipient rats in the control group at 7, 10, and 14 days posttransplant. The skin biopsies were approximately 3 × 3 mm and were harvested from ankles or feet of transplanted hindlimbs. These samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 16 hours at 4°C and cryoprotected for 24 hours in 20% sucrose in 0.1 M PB at 4°C. Thin sections (20 µm) of these skin samples were prepared. Hematoxylin and eosin stains of the skin samples from 7, 10, and 14 days posttransplant were obtained to assess the histological immunoreaction using the grading system for skin rejection. We randomly selected 2 slides from each sample, and 10 slides in the allograft group and 4 slides in the control group from each day were graded by 2 doctors in a blinded manner.

Immunohistochemistry

Skin biopsies were obtained from the same 5 recipients described above. The skin biopsies were fixed with 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 16 hours at 4 °C and cryoprotected for 24 hours in 20% sucrose in 0.1 M PB at
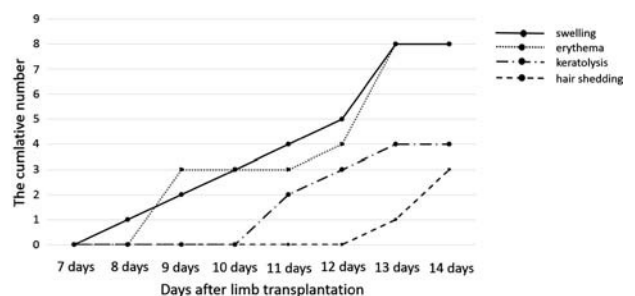

FIGURE 1. The change in the cumulative number of recipients with skin changes indicating acute rejection. All grafts showed swelling and erythema at 14 days and approximately half showed keratolysis and hair shedding.

The grading of hematoxylin-eosin–stained tissue sections at each time point

	Allograft group			Control group		
	7 days	10 days	14 days	7 days	10 days	14 days
Grade 0	7	6		3	4	3
Grade I	3	4		1		1
Grade II			2			
Grade III			8			

Most samples were classified grade 3 at 14 days in the allograft group.

4°C, after which the skin samples were cut into 20-μm-thick frozen sections using a cryostat. These sections were rehydrated in phosphate-buffered saline, and their antigens were retrieved with proteinase K (Sigma-Aldrich, St Louis, MO) at room temperature for 10 minutes, then the sections were incubated with blocking solution containing 2% donkey serum for 1 hour. Primary antibodies against CD4 (Bio-Rad AbD Serotec, Oxford, UK) and CD8 (BD Biosciences, Franklin Lakes, NJ) were added and incubated at 4°C overnight. The sections were then incubated in the appropriate fluorescent second antibody [CF543 (for CD4) and CF488 (for CD8) (Biotium, Richmond, CA)] for 1 hour at room temperature. We randomly selected 2 slides from each sample in the allograft group, assessed these randomly selected slides using light microscopy (Nikon D-Eclipse C1 confocal microscope; Nikon, Tokyo, Japan), and counted the number of stained cells.

Statistical Analysis

Data from immunohistochemistry and plasma miRNA measurements are presented as means and standard error. Statistical analysis was performed using with Excel 2013 and Student t test (Microsoft, WA), and the differences were considered significant at P less than 0.05.

RESULTS

Visual Skin Inspection

The cumulative numbers of recipients with skin changes are shown in Figure 1. After discontinuation of FK506, swelling and erythema of the allotransplanted hindlimb occurred gradually over several days. On day 14 posttransplant, all transplanted hindlimbs showed skin swelling and erythema. Keratolysis and hair shedding occurred subsequently; at 14 days, keratolysis had occurred in 4 rats and hair shedding in 3 rats of 8 recipients. By visual skin inspection, these changes occurred at a mean of 11.0 days after the surgery, ie, 5.0 days after discontinuation of immunosuppression.

Histological Study

The results of the histological assessment are shown in Table. Seven samples from 7 days posttransplant and 6 samples from 10 days posttransplant were classified as grade 0 in

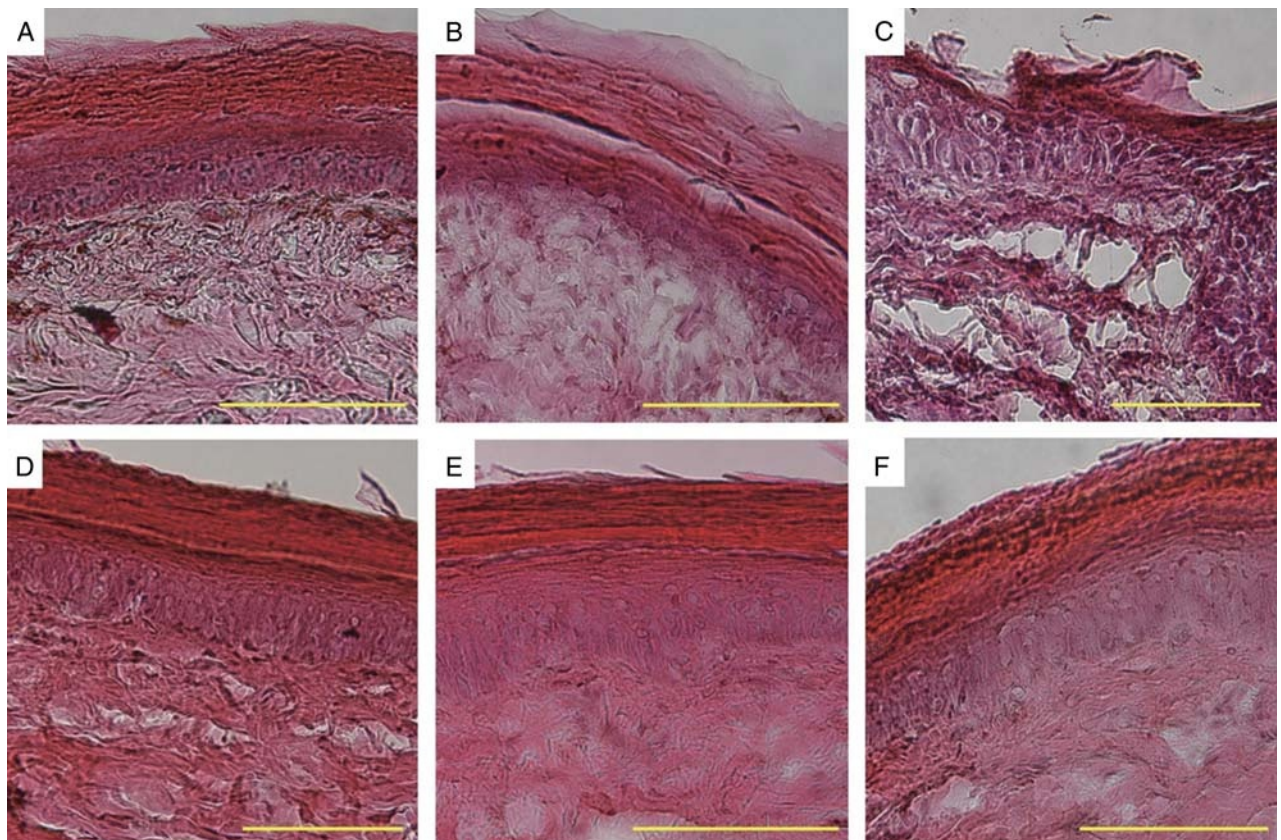**FIGURE 2.** Hematoxylin-eosin stain of skin biopsies from 7 (A), 10 (B), and 14 (C) days posttransplant in the allograft group and from 7 (D), 10 (E), and 14 (F) days posttransplant in the control group. A mild mononuclear cell infiltration was observed at 7 and 10 days posttransplant. Formation of many bullae and a major mononuclear cell infiltration were found at 14 days posttransplant. Scale bar, 100 μm.
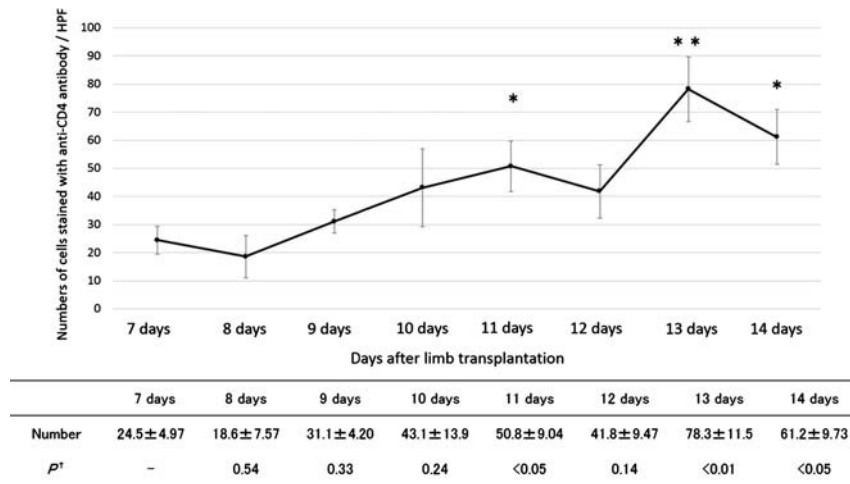
	7 days	8 days	9 days	10 days	11 days	12 days	13 days	14 days
Number	24.5±4.97	18.6±7.57	31.1±4.20	43.1±13.9	50.8±9.04	41.8±9.47	78.3±11.5	61.2±9.73
$P^{\dagger}$	–	0.54	0.33	0.24	<0.05	0.14	<0.01	<0.05

FIGURE 3. The change in numbers of cells stained with anti-CD4 antibody per high power field at each time point in the allograft group. The numbers at 11, 13, and 14 days posttransplant were significantly higher than that at 7 days posttransplant. † Each value was compared with the cell number at 7 days posttransplant. * $P < 0.05$. ** $P < 0.01$.

the allograft group, whereas 8 samples from 14 days posttransplant were classified as grade 3 and recognized as completely rejected. Histological analysis showed the formation of many bullae with an edematous appearance, a major infiltration by small mononuclear cells and epidermal necrosis 14 days after the surgery in the allograft group (Figure 2).

Immunohistochemistry Study

The mean numbers of CD4-positive cells at each time point in the allograft group are shown in Figure 3. The mean numbers of cells positive for CD4 antigen increased gradually after 8 days posttransplant (Figure 4). The numbers of

infiltrating CD4-positive cells at 11, 13, and 14 days posttransplant were significantly increased compared with that at 7 days posttransplant.

The mean numbers of CD8-positive cells also gradually increased from 8 days posttransplant in the allograft group. The numbers of cells stained for CD8 antigen at 13 and 14 days posttransplant were significantly increased compared with that at 7 days posttransplant (Figures 5 and 6).

miRNA

Based on previous reports, we selected 4 miRNAs (miRNA-146a, miRNA-155, miRNA-182, and miRNA-451) and
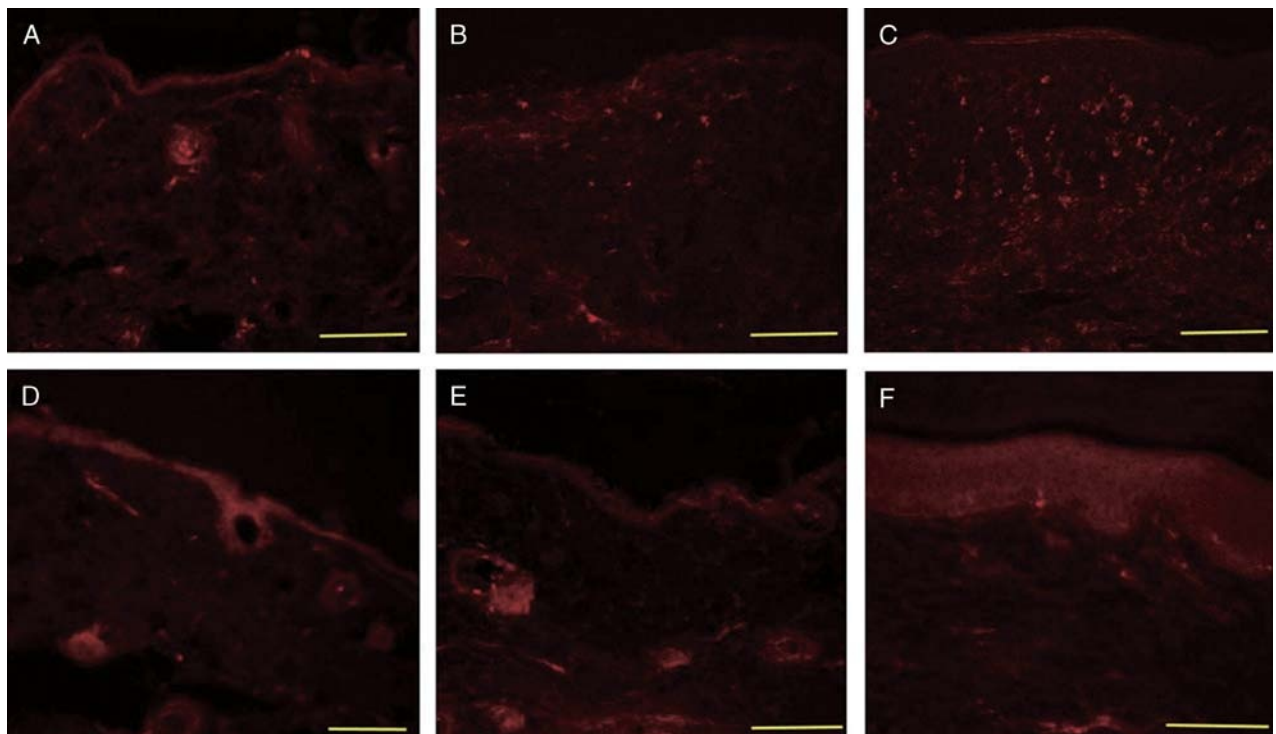

FIGURE 4. Immunohistochemistry study of CD4-positive cells in skin biopsies from 7 (A), 10 (B), and 14 (C) days posttransplant in the allograft group and from 7 (D), 10 (E), and 14(F) days posttransplant in the control group. Infiltration of CD4-positive cells gradually increased in the allograft group. Scale bar, 100 μm.
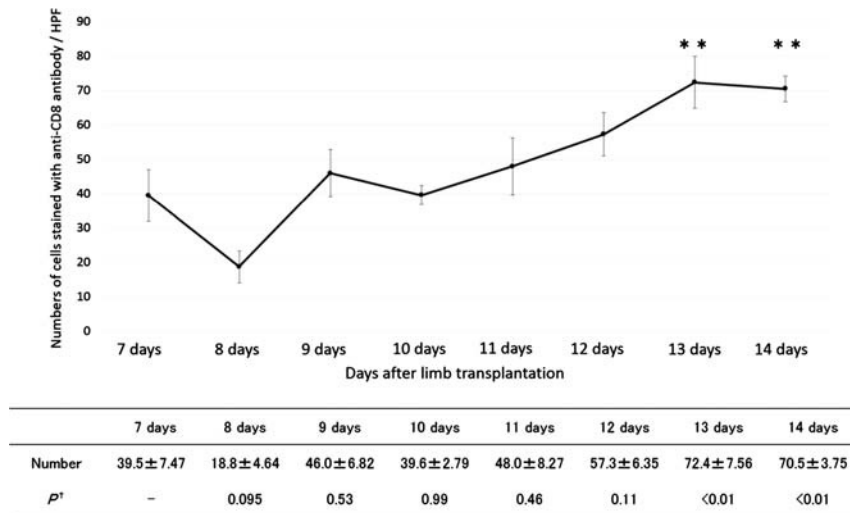
FIGURE 5. The change in numbers of cells stained with anti-CD8 antibody per high power field at each time point in the allograft group. The numbers at 13 and 14 days posttransplant were significantly higher than that at 7 days posttransplant. † Each value was compared with the cell number at 7 days posttransplant. * $P < 0.05$. ** $P < 0.01$.

	7 days	8 days	9 days	10 days	11 days	12 days	13 days	14 days
Number	39.5±7.47	18.8±4.64	46.0±6.82	39.6±2.79	48.0±8.27	57.3±6.35	72.4±7.56	70.5±3.75
$P^†$	–	0.095	0.53	0.99	0.46	0.11	<0.01	<0.01

determine their expression in plasma after limb allotransplantation.[22-24] Plasma miRNA-146a showed significantly higher expression at 10 and 14 days posttransplant than at 7 days posttransplant (increase = 2.10 ± 0.52 times, $P < 0.05$ at 10 days; 1.58 ± 0.30 times, $P < 0.01$ at 14 days). Plasma miRNA-155 was also significantly upregulated at 10 and 14 days posttransplant compared with that at 7 days posttransplant (increase = 2.51 ± 0.61 times, $P < 0.05$ at 10 days; 1.70 ± 0.34 times, $P < 0.05$ at 14 days) (Figure 7). Plasma miRNA-182 and miRNA-451 did not show significant changes.

The individual plasma expression changes of miRNA-146a and miRNA-155 are shown in Figure 8.

In control group, plasma expression at 10 and 14 days posttransplant showed no significant change compared with at 7 days posttransplant (Figure 9).

DISCUSSION

In limb transplantation, the majority of allotransplanted limbs survive despite their higher acute rejection rate compared
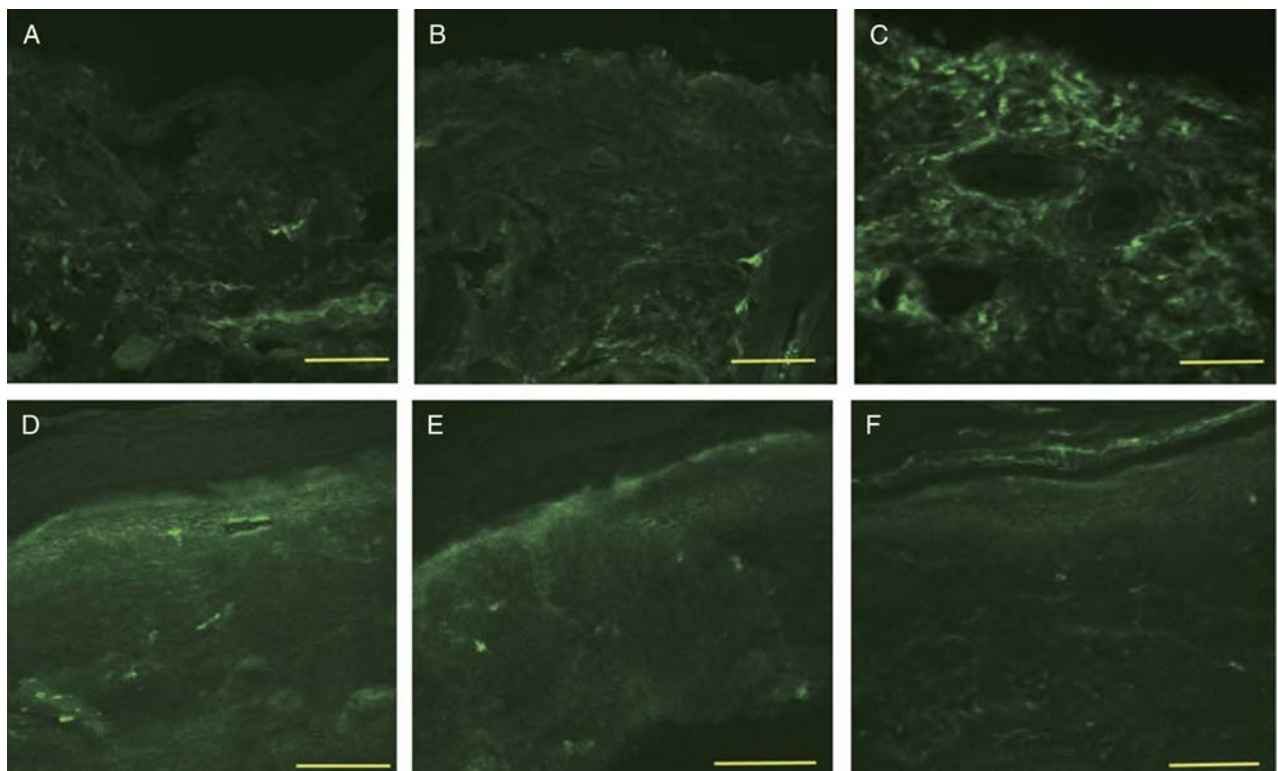

FIGURE 6. Immunohistochemistry study of CD8-positive cells in skin biopsies from 7 (A), 10 (B), and 14 (C) days posttransplant in the allograft group and from 7 (D), 10 (E), and 14(F) days posttransplant in the control group. Infiltration of CD8-positive cells gradually increased in the allograft group. Scale bar, 100 μm.
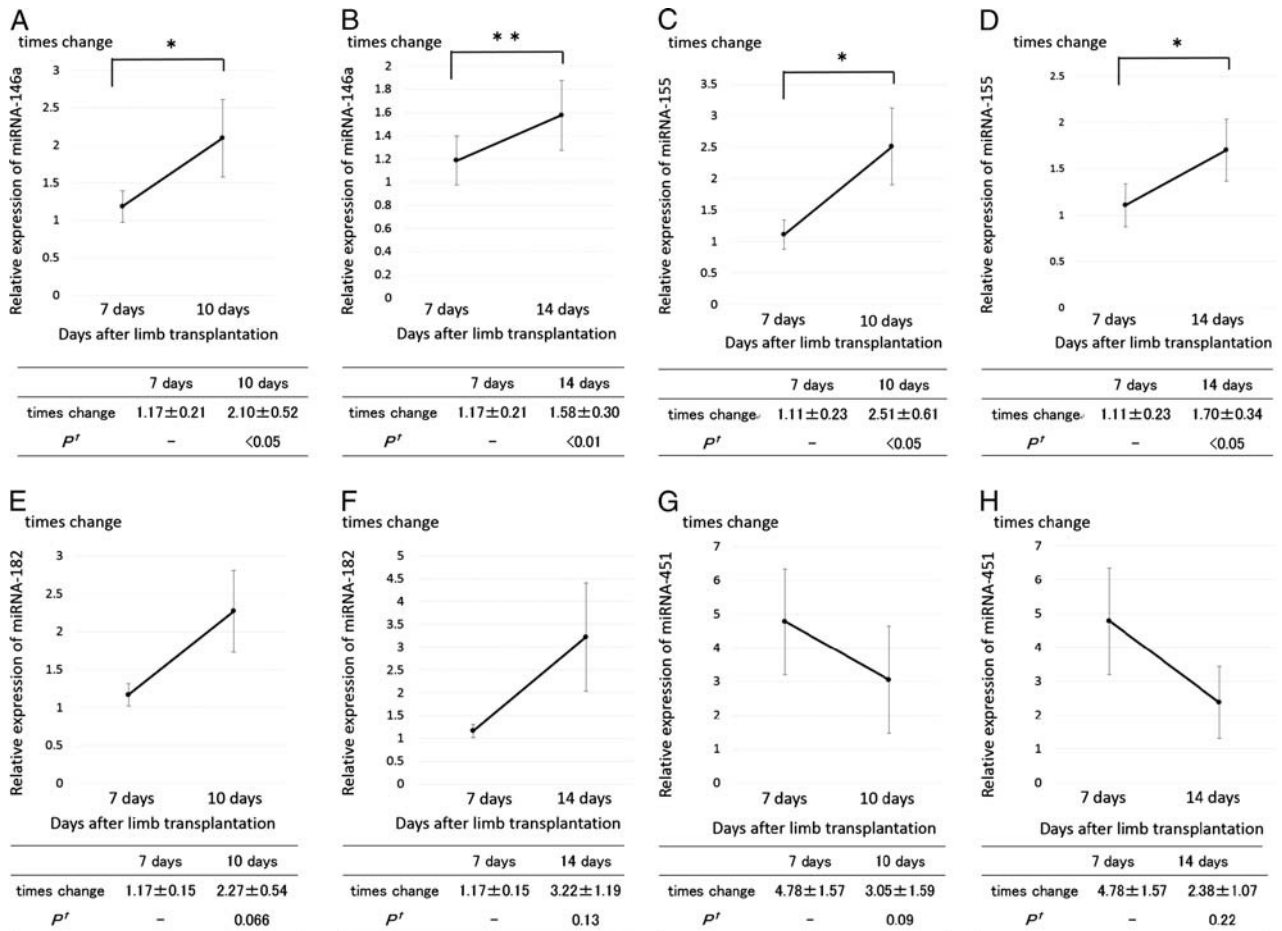
FIGURE 7. The change in plasma expression of miRNA-146a (A, B), miRNA-155 (C, D), miRNA-182 (E, F), and miRNA-451 (G, H) in allograft group. Plasma expression of miRNA-146a at 10 and 14 days posttransplant was significantly upregulated compared with that at 7 days posttransplant. Plasma miRNA-155 expression at 10 and 14 days posttransplant was also significantly higher than that at 7 days posttransplant. Plasma expression of miRNA-182 and miRNA-451 did not show significant postoperative changes. † Each value was compared with the expression at 7 days posttransplant. *$P < 0.05$. **$P < 0.01$.

with that for kidney transplantation.[1] However, as is the case for renal transplantation, unrecognized subclinical acute rejection can cause graft deterioration resulting in late graft loss.[1,25,26] A prospective study showed the importance of early diagnosis and treatment of acute rejection that prevented the deterioration of renal transplants.[27] Thus, in renal transplantation, it is important to diagnose acute rejection and treat it early. It is considered that mild acute rejection in an allotransplanted limb could gradually damage several types of tissue causing their dysfunctions and eventually graft loss. Earlier recognition and earlier treatment for acute rejection are as important in limb allotransplantation as in renal transplantation. Currently, visual skin inspection and histological evaluation are used to assess the rejection of hand transplants, but problems exist because these evaluations are largely subjective methods, and in addition, biopsies are invasive procedures for transplant recipients.
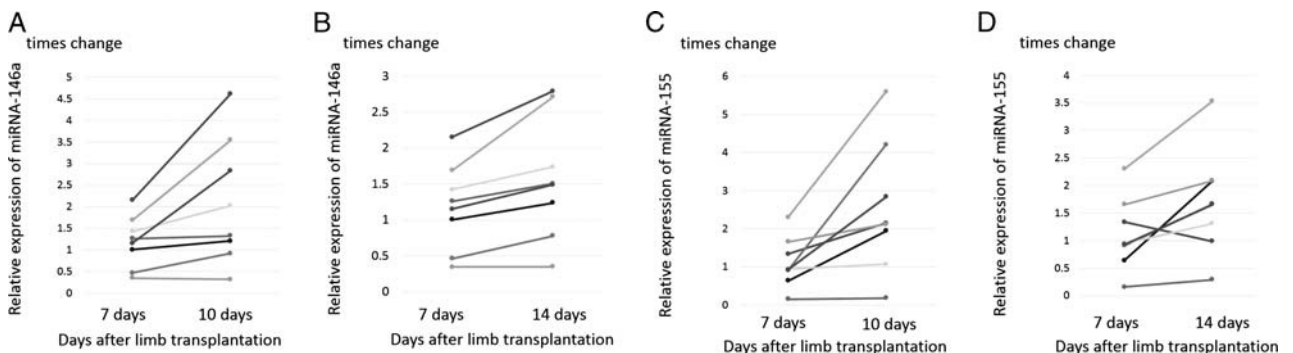

FIGURE 8. The Individual change in plasma expression of miRNA-146a (A, B), miRNA-155 (C, D) in allograft group. Almost all sample show plasma expression increase at 10 and 14 days posttransplant compared with that at 7 days posttransplant in both miRNAs.
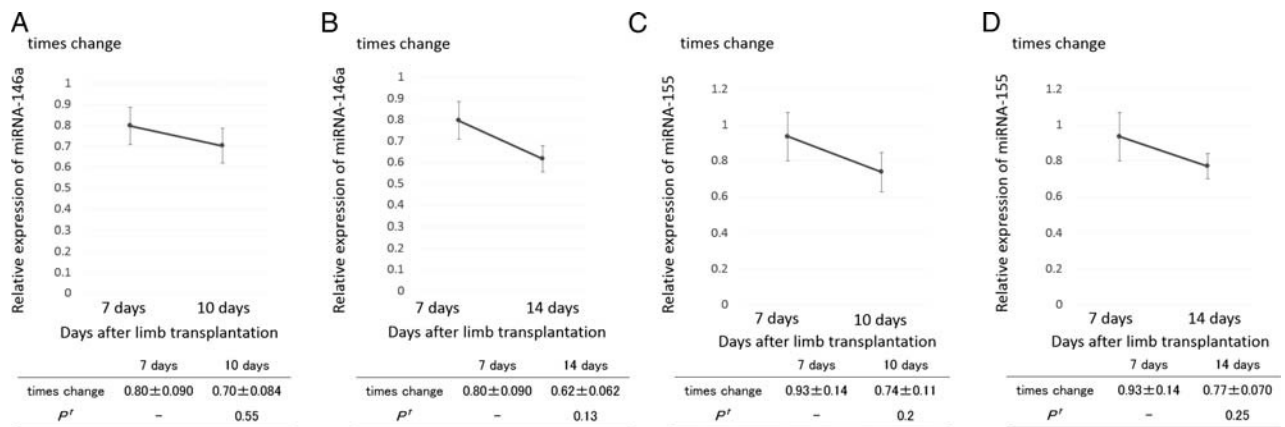
FIGURE 9. The change in plasma expression of miRNA-146a (A, B), miRNA-155 (C, D) in control group. In both miRNAs, plasma expression at 10 and 14 days posttransplant was not significantly changed compared with that at 7 days posttransplant. † Each value was compared with the expression at 7 days posttransplant.

Mature miRNAs are noncoding RNAs that are important factors in immune responses, control of homeostasis, and cellular development.[10,14,28] They have recently been recognized as biomarkers for several diseases including cancer or autoimmune diseases.[10,21] Some authors have reported variation of the expression of microRNAs in plasma or serum during acute rejection after organ transplantation, and noted that they could serve as a minimally invasive diagnostic tool.[3,15] However, there is little recognition or understanding of the role of miRNAs in acute rejection of limb transplantation, even in rats.

In this study, we showed that expression of miRNA-146a and miRNA-155 in plasma is upregulated at 10 days posttransplant, that is, by 4 days after discontinuation of immunosuppressive therapy. In contrast, skin changes recognized as acute rejection appeared at a mean of 11.0 days posttransplant. By histological analysis, more than half of all slides from 10 days posttransplant were classified as grade 0 for rejection, and none were assessed as grade 3 indicating complete rejection. Therefore, we demonstrated that changes in plasma miRNA-146a and miRNA-155 expression indicate acute rejection earlier than the visual or histological appearance.

miRNA-146a is rapidly induced by TNF-α, IL-1β, lipopolysaccharide (LPS), and other immune stimuli and is differently expressed in some autoimmune diseases.[29-31] It is thought to play important roles in proinflammatory signaling and in the differentiation or maintenance of CD4+ T regulatory cells[31,32] miRNA-155 is induced by TNF-α, IFN-γ, and toll-like receptor ligands such as bacterial LPS. It activates several types of immune cells resulting in enhanced secretion of various cytokines.[10] miRNA-155 is also reported as a component of the primary macrophage response[11] and to be overexpressed in autoimmune diseases and cancers.[10,11] As outlined above, miRNA-146a and miRNA-155 have multiple functions in the immune system, and both are overexpressed in some diseases[29,32] and in acute rejection in a mouse-to-rat cardiac xenotransplantation model.[22] Because both these miRNAs are overexpressed in various diseases and are induced by various immune stimuli,[14,29,31] we can speculate that they are upregulated in various situations such as infection, graft rejection or surgical invasion. In this study, the control group showed no change in expression between 7 days posttransplant and 10 and 14 days posttransplant. Therefore, we can speculate that the changes in expression of these 2 plasma miRNAs in allograft group in this study reflected acute rejection of the transplanted limbs and were not caused only by the stresses of surgery or blood sampling. These miRNAs are probably functioning in the early and critical stages of immune reactions and immune regulation, and therefore their expression changes earlier than parameters assessed by visual skin inspection and histological assessment. Indeed, in this study, changes in the expression of plasma miRNA-146a and miRNA-155 were recognized before tissue damage occurred macroscopically or microscopically. They could therefore be potential biomarkers for the early recognition of acute rejection. Repetitive minor rejections gradually damage graft function and can result in graft loss.[25-27] Therefore, avoiding minor rejection after limb transplantation seems to be important. The detection of rejection before tissue damage occurs can allow early changes of immunosuppressive medication that can maintain graft function and reduce the rate of limb transplantation rejection.

In this study, we applied the same protocol to all samples. However, the extent of acute rejection differed between samples, as seen in Figure 1 and Table. This might be caused by the timing and leakage of immune suppressive drugs or weight changes after transplantation. We can speculate that this is the reason for the wide variation in the expression of miRNAs in plasma. There was 1 rat whose plasma expression of miRNA-155 decreased at 14 days posttransplant compared with that at 7 days posttransplant. In this case, keratolysis was already seen at 11 days posttransplant. Therefore, we can infer that the peak of plasma expression had already passed by 14 days posttransplant. Wang et al[33] reported that in a mouse model of sepsis, expression of miRNA-155 peaked at 12 hours and returned to baseline within 2 days after LPS exposure despite prolonged expression of some cytokines and enzyme. However, miRNA-146a plasma expression in all rats increased at 14 days posttransplant compared with that at 7 days posttransplant.

This study has some limitations. First, as noted, miRNA-146a and miRNA-155 are overexpressed in a number of diseases or inflammatory responses, and therefore may be considered to have low specificity and be unsuitable for differential diagnosis. Further investigation is needed to clarify

their specificity. We propose that analysis of a combination of various miRNAs could allow an accurate diagnosis with high specificity and high sensitivity. Second, rat miRNAs differ from human miRNAs. For application to human hand transplantation, it is necessary to investigate a range of human miRNAs. Nevertheless, plasma miRNA expression is a potential biomarker for diagnosis of acute rejection in composite tissue allotransplantation in a similar manner to transplantation of other organs.

CONCLUSIONS

We conclude that miRNA-146a and miRNA-155 expression in plasma could act as a biomarker for acute rejection of allotransplanted limbs and allow earlier diagnosis. This method is less invasive than tissue biopsy. Early diagnosis and early treatment for acute rejection of allotransplanted limbs may improve graft function and prognosis.

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