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Increased Rac2 mRNA expression in peripheral blood during human corneal graft rejection

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LABORATORY STUDY

Abstract

Purpose **Allograft rejection is the main cause of graft failure in human corneal transplantation, for which underlying pathomechanism is not yet clear. We compared gene expression in the peripheral blood of patients who after undergoing corneal transplantation experienced graft rejection with those patients who accepted grafts.**
Methods **Sixty-six patients who underwent corneal transplantation were studied including 18 patients who suffered subsequent graft rejection. cDNA array technology was used to survey and quantify transcript expression . A semiquantitative reverse transcriptase–PCR (RT–PCR) was used to confirm the gene expression pattern measured by a cDNA array of selected genes.**
Results **Among 265 genes present on the array, eight genes were found to be differentially expressed. Four genes (*Rac 2*, *RhoA*, *paxillin*, and *CD18*) were further analysed by semiquantitative RT–PCR, and significant differences in mRNA expression levels in the rejection group were confirmed.**
Conclusions **Our study demonstrated that the expression of *Rac2* mRNA was upregulated in the peripheral blood of patients experiencing corneal transplantation rejection compared to those patients who had no rejection episodes. In addition, three genes, *RhoA*, *paxillin*, and *CD18*, showed decreased expression in rejecting patients. cDNA array technology provides a potentially useful approach to identify novel genes that might participate in pathogenic pathways during corneal graft rejection.**
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Introduction

The corneal transplant survival rate is as high as 90% in the first year after surgery. The most common cause of transplant failure is allograft rejection, which accounts for 20% of patients following penetrating keratoplasty at 10 years postoperatively.[1] It had been thought that the relatively high acceptance of corneal grafts was because of the immune privilege of the cornea attributed largely to its avascularity. It is now clear that several factors contribute to defects in immune privileg, such as a breakdown of the blood–ocular barrier, loss of inhibitory factors in the aqueous humour, abrogation of anterior chamber-associated immune deviation (ACAID), and the loss of FasL expression within the anterior chamber. In an earlier study,[2] a reduction in Fas mRNA expression in blood samples was seen during episodes of graft rejection. Levels returned to normal after resolution of the episode. These preliminary data indicated that markers of corneal graft rejection can be detected in the peripheral circulation.[3]

During corneal graft rejection, inflammatory cells, including T lymphocytes, neutrophils, natural killer cells, dendritic cells, and macrophages, infiltrate the graft.[4–8] Adhesion molecules on cell surfaces allow inflammatory cells, present in the circulation to adhere to the vascular cell wall and migrate through the vessel wall into the rejecting tissue. Several adhesion molecules, including LFA-1 and ICAM, have been implicated in corneal graft rejection.[9–11] In this study, we used array

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technology to investigate the changes in the expression of genes involved in cell interactions and adhesion in peripheral blood at the time of corneal graft rejection episodes. Identifying such genes may further advance our knowledge of the processes involved in graft rejection and provide useful prognostic markers of graft rejection

Materials and methods

Subject

Sixty-six patients who underwent a corneal transplantation at the Manchester Royal Eye Hospital were recruited. This included 18 patients who experienced a first episode of corneal transplant rejection in either eye. The diagnostic criteria for graft rejection were (a) development of an epithelial or endothelial rejection line, (b) an anterior chamber reaction with keratic precipitates in a previously clear graft, and (c) increasing corneal stromal oedema and visible aqueous cells. Patients were categorized as being at high risk, if they had one of the following risk factors: two or more quadrants of corneal stromal vascularization, previous graft rejection, concurrent intraocular inflammation, anterior synechiae, or a history of chronic

intraocular inflammation. The mean follow-up period for these patients was 30.8 months. Blood samples were taken from recipients at the time of transplantation, at subsequent clinic visits and during and after episodes of graft rejection. The samples used to represent the rejecting patients were taken during episodes of rejection except in the longitudinal study when additional samples were taken at times when no rejection was occurring. Blood samples were also collected from 30 normal controls. The demographics of the subjects studied are summarized in Table 1 and the details of patients experiencing rejection are given in Table 2.

We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. The collation of human samples was approved by Salford and Trafford NHS Research Ethics Committee (Project Reference 05/Q1404/129) and the University of Manchester Ethics Committee (ref 05307).

RNA extraction

Total RNA was isolated from blood samples using the trizol reagent (Invitrogen, Paisley, UK). Briefly, 200 μl of

Table 1 Demographics of corneal graft patients and normal controls

Subjects	Number	Mean age at the time of graft (years)	Age range	High risk
Graft recipients—rejection	18	51.2	16~82	50%
Graft recipients—no rejection	48	57.9	17~100	47.9%
Normal controls	30	35	18~72	NA

Table 2 Corneal graft patients undergoing episodes of rejection

Patient	Age at the time of graft (years)	Diagnosis	Risk of rejection	Time of rejection (months after graft)	Treatment	Outcome
CG-2	26	Keratoconus	Low	2	TS	Resolved
CG-7	79	Interstitial keratitis	Low	6	TS	Resolved
CG-9	45	HSK+ microbial infection	High	6.5	TS	Failed
CG-21	82	HSK/VZV	High	11.5	TS	Resolved
CG-30	74	Microbial keratitis	High	42.5	TS+i.v.	Resolved
CG-46	69	Fuchs' dystrophy	Low	61	TS	Failed
CG-47	75	HSK	High	32	TS	Resolved
GG-52	68	HSK	High	38	TS	Resolved
CG-65	16	Anterior chamber dysgenesis	High	12	TS	Resolved
CG-103	72	Previous failed graft for HSK	High	33	TS	Resolved
CG-133	69	Old injury with vascularization	High	8.5	TS	Resolved
CG-151	74	Pseudophakic bullous keratopathy	Low	8.5	TS	Resolved
CG-155	29	Keratoconus	Low	2.5	TS	Resolved
CG-258	26	Keratoconus	Low	12	TS	Resolved
CG-259	22	Keratoconus	Low	9	TS	Resolved
CG-264	18	Keratoconus	Low	5	TS	Resolved
CG-278	23	Keratoconus	Low	44	TS+i.v.	Resolved
G-303	56	Previous failed graft for aniridia	High	14	TS	Resolved

i.v. = intravenous steroids; TS = topical steroids.

fresh blood was mixed with 800 μl Trizol reagent and incubated at room temperature for 10 min. After chloroform (200 μl) was added, the sample was incubated at room temperature for 2∼3 min, and centrifuged at 13 000 × g at 4°C for 15 min. The supernatant was removed, and the pellet was resuspended in 500 μl isopropanol. The tube was centrifuged again for 10 min. The pellet was washed twice with 75% ethanol and centrifuged at 10 000 × g at 4°C for 10 min. The final RNA pellet was resuspended in 19 μl of DEPC water, and 1 μl of RNase inhibitor was added. The sample was aliquoted and immediately frozen at −80°C.

Analysis of gene expression using the Atlas human cDNA array

Atlas^TM human cell interaction cDNA arrays containing 265 genes (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) were probed with cDNA produced from three rejecting (taken at the time of rejection) and three non-rejecting patients. Briefly, the extracted RNA was treated with RNase-free DNase for purification. First-strand cDNA synthesis was performed using the SMART PCR cDNA synthesis kit (Clontech-Takara Bio Europe). Long-distance PCR was performed for cDNA amplification using the Advantage 2 PCR kit (Clontech-Takara Bio Europe), and the number of PCR cycles was optimized. All procedures were performed according to the manufacturer's instructions. ^{32}P-labelled cDNA was then synthesized from the purified PCR products using reagents provided with the array (cDNA synthesis (CDS) primer mix, labelling buffer, dNTP mix (Clontech-Takara Bio Europe), and [α-^{32}P]dATP (GE Healthcare, Buckinghamshire, UK)). Hybridization of the purified radioactively labelled cDNA to the cell interaction array membrane and subsequent washes were performed according to the user's manual. The nylon membrane array was exposed to autoradiographic film (Biomax MS, Sigma, Dorset, UK), and the expression pattern was visualized. The data were analysed using AtlasImage 2.0 software (Clontech-Takara Bio Europe), and the results were normalized with reference to the housekeeping gene β-actin.

Reverse transcriptase–PCR (RT–PCR)

The gene expression profiles detected by array hybridization were independently confirmed for the selected gene by semiquantitative RT–PCR. An RT step (42°C for 30 min, 95°C for 5 min, and 4°C for 5 min) followed by a PCR was performed. Ready-to-go RT–PCR Beads^TM (Amersham Pharmacia Biotech, Bucks, UK) containing MMLV reverse transcriptase, 10 mM Tris-HCl (pH 9.0), 6.0 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM of each dNTP, and 2 U *Taq* DNA polymerase were used for the RT–PCRs in a 50-μl reaction volume. The primers and setup for the RT–PCR are described in Table 3. Amplified products were resolved by 1% (w/v) agarose gel electrophoresis on a gel-containing ethidium bromide in 1 × TBE buffer at 10 V/cm for 1 h. The level of selected gene expression was determined by comparison to expression of a housekeeping gene (β-actin) by gel densitometry using the GeneTools gel analysis package (Syngene, Cambridge, UK). This semiquantitative measurement was represented as the ratio of the gene of interest to β-actin. One of the control samples was used as a standard and added to each RT–PCR/gel run.

Table 3 PCR primers, fragment size, and cycling parameters for the gene expression assays

Gene	GenBank accession number	Primers (5'-3')	Fragment size (bp)	Cycles
β-Actin	X00351	CGTTGCTATCCAGGCTGTGC GTAGTTTCGTGGATGCCACA	434	25 cycles (95°C for 45 s, 57°C for 60 s, and 72°C for 45 s)
Rac2	M29871	GCAAGACCTGCCTTCTCATCAGC CTCGATGGTGTCCTTGTCGTCCC	338	35 cycles (95°C for 60 s, 57°C for 60 s, and 72°C for 60 s)
RhoA	L25080	CCGCCTTCGTCTCCGAGTTTGCGAC GATAAATGAAAAAGGCCAGTAATCATACACTAAG	752	35 cycles (95°C for 45 s, 57°C for 60 s, and 72°C for 45 s)
CD18	M15395	CATCACTGTGATGGCCATGGC CATGGGACCCTGCAGATTCTG	385	35 cycles (95°C for 45 s, 61°C for 60 s, and 72°C for 45 s)
Paxillin	U14588	GGAATGCTTCACGCCATTCG CTATCAAAGTCGGACAGTGTC	957	30 cycles (95°C for 60 s, 57°C for 60 s, and 72°C for 60 s)

This allowed comparisons between samples run on different gels.

Statistical methods

A one-tailed Student's *t*-test for two samples assuming unequal variances was used to determine the significance of the frequency difference between these groups. Results were considered significant at $P < 0.05$.

Results

Analysis of differential mRNA expression by cDNA array

To investigate the differences in gene expression between rejecting and non-rejecting patients, the cell interaction cDNA arrays were probed. Following normalization, the probed arrays showed that several genes were expressed on the cell interaction array. Most genes were expressed at equal levels by both rejecting and non-rejecting patients. A comparison of the autoradiographs from the two arrays showed that several genes were differentially expressed between rejecting and non-rejecting patients (Table 4). In this study, four genes were selected for further analysis; *Rac2*, which showed an increase in expression in the rejecting patient group, and *CD18*, *RhoA*, and *paxillin*, which all showed decreased expression in the rejecting patient group.

Confirmation of array results by semiquantitative RT–PCR

To confirm that the changes in mRNA expression were representative of all patients who experienced corneal graft rejection and that these expression changes were not just specific to the patients whose blood was used to probe the arrays, semiquantitative RT–PCR assays were designed. These semiquantitative RT–PCR assays were optimized to allow a group of rejecting patients to be compared to both normal controls and corneal graft patients who did not experience rejection. Gene-specific primers were used on the total RNA extracted from whole blood as a target. A housekeeping gene (*β*-actin) was amplified as an internal control to produce a 434-bp product. Figure 1 shows the varying levels of mRNA expression for the four genes in the patient and control groups. mRNA levels were expressed as the ratio of the target gene to *β*-actin mRNA.

The RT–PCR results showed that *Rac2* levels increase significantly in samples taken at the time of a rejection episode from rejecting patients (mean = 0.891, SEM = 0.034) compared with those from normal controls (mean = 0.65, SEM = 0.0009, $P = 0.0001$), or corneal

Table 4 Cell interaction-related genes expressed in the blood of corneal transplantation patients

	GenBank accession number	Change in expression
Genes expressed equally		
Caveolin 1	Z18951	—
Caveolin 2	AF035752	—
CD120B antigen	M32315	—
Ephrin B3	U66406	—
Ephrin type-A receptor 2	M59371	—
Ephrin-B1	U09304	—
E-selectin	M30640	—
Fibronectin 1 (FN1)	X02761	—
Frizzled homologue 9 (FZD9)	U82169	—
Integrin alpha L (ITGAL)	Y00796	—
jagged homolog 2 (JAG2)	AF003521	—
L1 cell adhesion molecule (L1CAM)	M74387	—
Laminin receptor 1	U43901	—
NOTCH 1	M73980	—
Plasminogen (PLG)	X05199	—
Rac1	M29870	—
Rho–GDP dissociation Inhibitor 1	X69550	—
Rho-related GTP-binding protein	X61587	—
Tissue inhibitor of metalloproteinase 1	X03124	—
Genes which increased during graft rejection		
Collagen VIα3 subunit (COL6A3)	X52022	$2.5 \times$ [a]
Rho–GDP dissociation inhibitor 2	L20688	$2 \times$ [a]
Rac2	M29871	$11 \times$ [a]
Genes, which decreased during graft rejection		
Platelet-activating factor receptor (PAFR)	M54995	$12.5 \times$ [b]
Integrin *β*2 (ITGB2) or (*CD18*)	M15395	$2 \times$ [b]
RhoA	L25080	$10 \times$ [b]
Paxillin (PXN)	U14588	$3.3 \times$ [b]

Differences in gene expressions between rejecting and non-rejecting patients are shown.
[a]Increase in rejecter compared to non rejecters (rejecter/non rejecters).
[b]Decrease in rejecter compared to non rejecters (non rejecter/rejecters).

transplant recipients not experiencing graft rejection (mean = 0.71, SEM = 0.013, $P = 0.0013$). The other three genes studied showed decreased levels of expression at the time of rejection compared to non-rejecting patients or normal controls. *RhoA* showed a significant decrease in expression in rejecting patients (mean = 0.14, SEM = 0.01) *vs* non-rejecting patients (mean = 0.39, SEM = 0.07, $P = 0.000006$) and controls (mean = 0.34, SEM = 0.036, $P = 0.000017$). *Paxillin* showed a significant decrease in expression in rejecting patients (mean = 0.69, SEM = 0.23) compared to non-rejecting patients (mean = 0.95, SEM = 0.14, $P = 0.041$) and controls (mean = 1.03, SEM = 0.19, $P = 0.017$). *CD18* showed a significant decrease in expression in rejecting patients (mean = 0.73, SEM = 0.053) *vs* non-rejecting patients
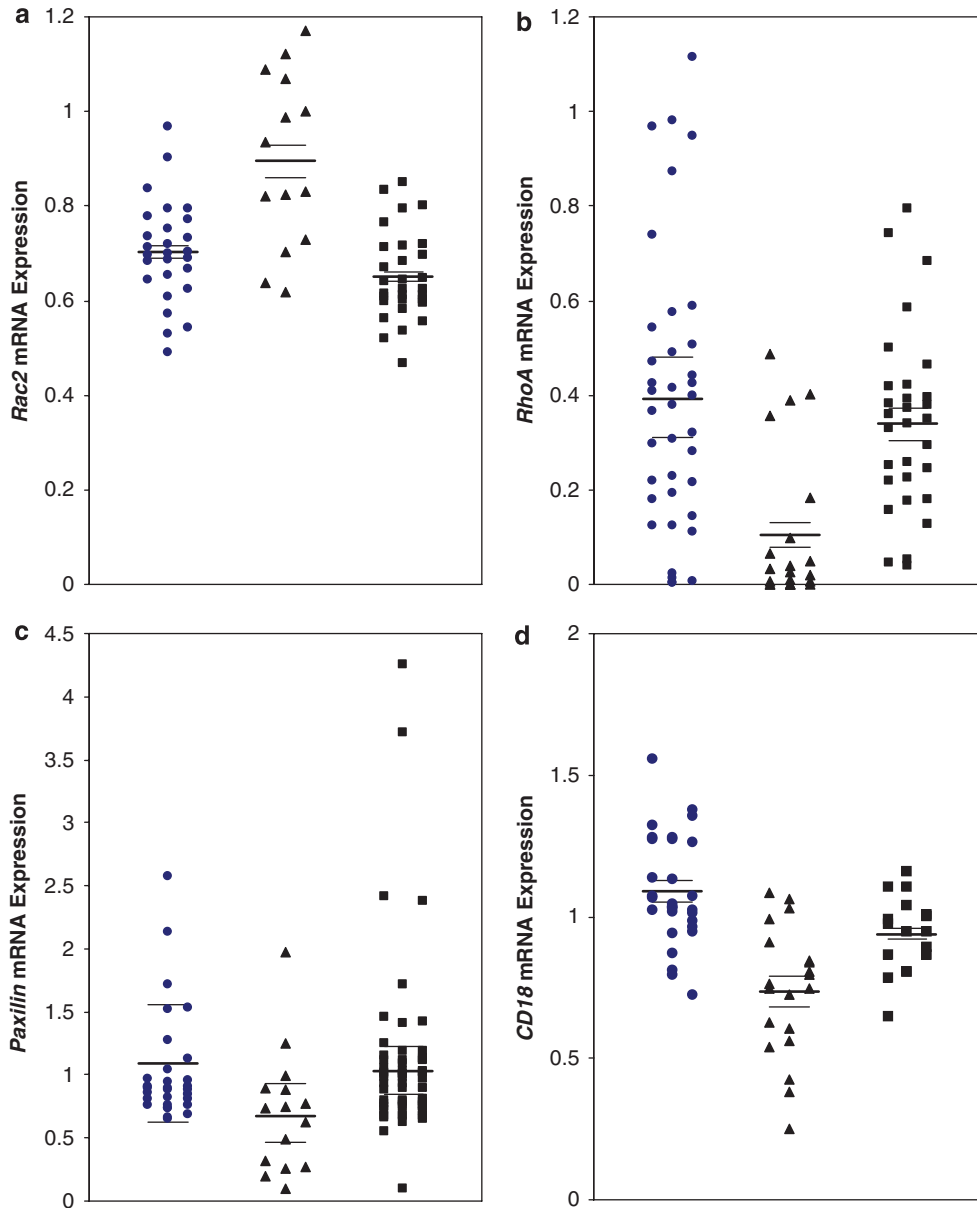
Figure 1 mRNA expression in blood samples from corneal graft patients and controls. Three groups of patients were studied: corneal graft patients who had not suffered an episode of graft rejection (●), corneal graft patients suffering an episode of graft rejection (▲), and normal controls (■). Four genes were measured, and levels are shown as a ratio of gene expression compared with β-actin. (a) *Rac2* showed a significant increase in expression in rejecting patients *vs* non-rejecting patients and controls. (b) *RhoA*, (c) *paxillin* and (d) *CD18* all showed a significant decrease in expression in rejecting patients *vs* non-rejecting patients and controls. (—: mean, −: SEM).

(mean = 1.09, SEM = 0.036, $P = 0.000001$ and controls (mean = 0.94, SEM = 0.18, $P = 0.0011$).

Serial samples were available for several patients who suffered an episode of graft rejection; some of them included samples before and after the rejection episode and from patients who had not suffered from rejection. *Rac2* mRNA levels of rejection patients are represented in Figure 2a and non-rejection patients in Figure 2b. Levels of *Rac2* mRNA at the time of graft rejection (mean = 1.01) were significantly higher than those taken from this group when they where not experiencing a rejection episode (mean = 0.81, $P = 0.0077$). There was no significant difference between samples taken several months apart in the patient group, which did not experience rejection (mean = 0.73). This longitudinal study also showed that when samples taken from
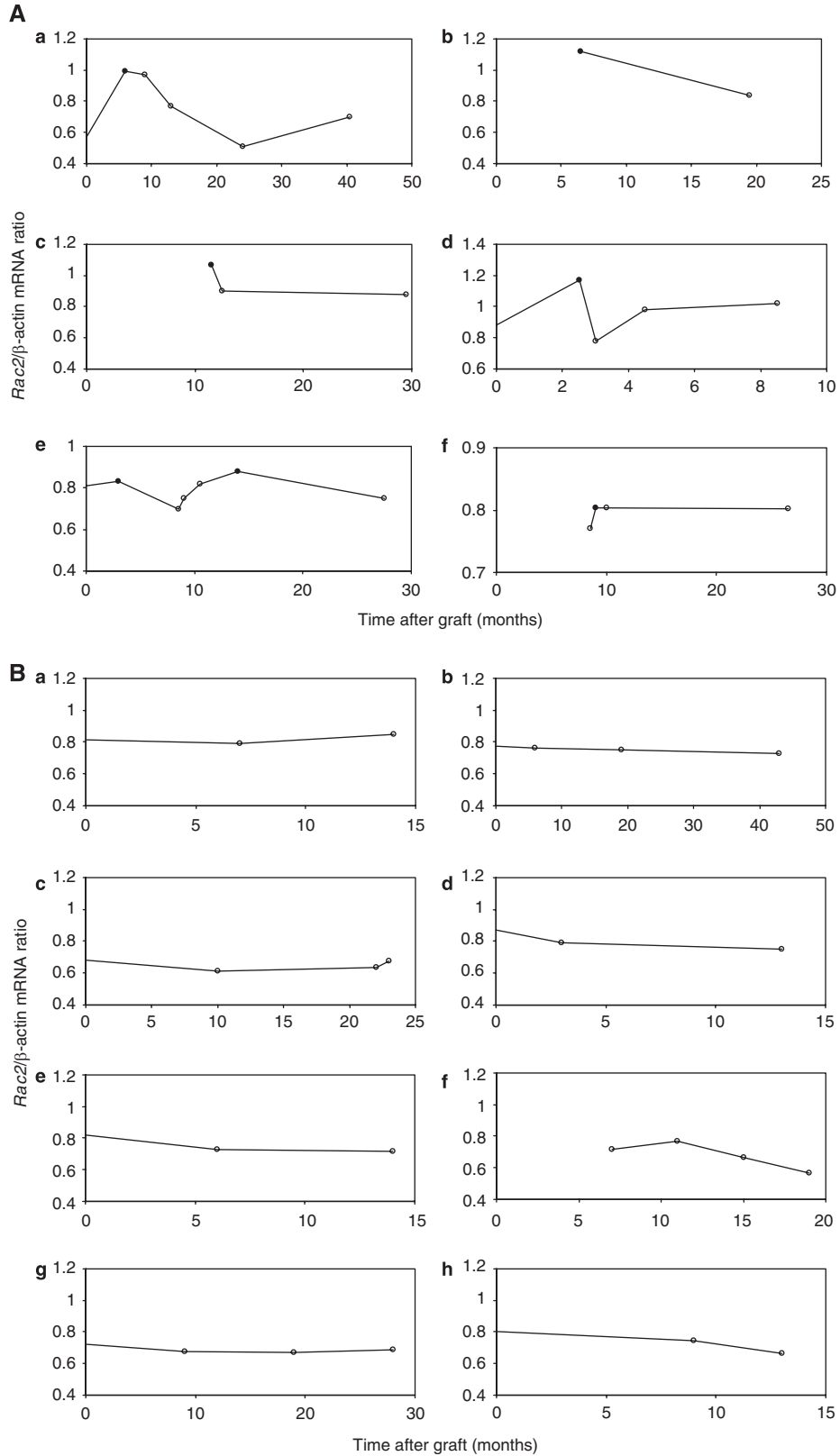
Figure 2 A longitudinal study of *Rac2* mRNA expression in patients who suffered an episode of graft rejection (A) and patients who had suffered no rejection episode (B). ● Indicates the time of the episode of graft rejection and ○ indicates that no episode of rejection occurred.

rejecting patients, after the rejection episode had been resolved, were compared to those of non-rejection patients, no significant difference was seen.

Discussion

Using cDNA array analysis, a comparison was made between the gene expression profiles of blood samples obtained from corneal transplant patients experiencing graft rejection and those who did not reject the transplant. Several genes that were differentially expressed have been identified, and four of these genes were selected for further analysis. The genes were chosen because they are involved in processes, which are associated with controlling the immune system. *Rac2* codes for a low-molecular weight guanosine triphosphate (GTP)-binding protein and has been shown to play a role in pathways controlling the growth and death of T cells.[12] Paxillin is a focal adhesion protein that recruits adhesion and growth factor-mediated signals from the extracellular matrix and is expressed at high levels in circulating lymphocytes and macrophages.[13–15] RhoA, together with the rest of the proteins that form the Rho superfamily, is involved in signal transduction, proliferation, vesicle trafficking, cell cycle progression, gene expression, and regulation of the actin cytoskeleton.[16] CD18 is a subunit of two separate β2-integrins; LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), both of which are expressed on inflammatory cells and are counter ligands for ICAM-1. LFA-1 in conjunction with ICAM-1 has been shown to be important in animal models of corneal graft rejection, as monoclonal antibodies blocking LFA-1 reduces the occurrence of graft rejection.[10]

From further analysis of the selected genes, only *Rac2* exhibited significantly higher levels of mRNA during corneal graft rejection episodes compared to non-rejecting patients. Semiquantitative RT–PCR further confirmed that there were significant differences between rejection and non-rejection groups.

Acceptance of a corneal graft relies on multiple contributory factors including the immune privilege of the recipient eye as well as the quality of the donor cornea. Therefore, we could not exclude the possibility that the difference between rejection and non-rejection groups was because of an underlying pathomechanism of each individual. It is reasonable to hypothesize that increased Rac2 expression is one of the factors involved in corneal graft failure as Rac2 had been demonstrated to play a central role in T helper 1 (Th1), cell differentiation, and cytokine production.[17] Evidence from studies in mouse and rat models have shown the predominance of Th1 cytokine expression in cells infiltrating the graft during corneal allograft rejection.[18–20] The Th1 cytokine,

interferon-γ, as well as other cytokines, such as IL-1β, IL-2, IL-12, have also been shown to compromise ocular immune privilege, and their upregulation may contribute to graft rejection.[18,21] Recently, anti-interferon-γ antibodies were reported to be effective in halting corneal transplant rejection after penetrating keratoplasty.[22] Rac2 has previously been demonstrated both *in vitro* and *in vivo* to be required for interferon-γ production,[23] and in turn may indirectly contribute to graft rejection.[17] It is unclear whether elevation of the *Rac2* gene contributes to graft rejection or whether it is a consequence of the rejection. However, the significant difference in *Rac2* gene expression between rejecting and non-rejecting patients suggests its role in corneal graft rejection. Levels of *Rac2* mRNA in patients undergoing corneal transplantation either with or without graft rejection were significantly higher than the normal controls in our study. It is possible that as a result of the surgical procedure, *Rac2* gene expression is activated because of allogeneic reactions. It was also noted that after treatment, *Rac2* gene expression levels were reduced and even returned to normal in several cases, as revealed in the longitudinal study. This might partly have resulted from the effect of glucocorticoids or other immunosuppressive agents used in the treatment to resolve grafts. It is well known that glucocorticoids suppress the cell-mediated immune response and inhibit the secretion of Th1,[24,25] although it is still too soon to say whether Rac2 signalling is involved in this suppression.

The decrease in expression of the other three genes is harder to explain, especially CD18, which is a subunit of LFA-1, which known to be expressed on lymphocytes and binds to ICAM-1 on the corneal endothelium allowing infiltration and rejection. In contrast to this study, LFA-1 was shown to be active in a murine model of corneal graft rejection.[10] They showed that the administration of anti-LFA-1 antibodies reduced the incidence of graft rejection from 90% in untreated mice to 47% in anti-LFA-1-treated mice.[10] It is possible that *CD18* mRNA levels do not represent levels of the LFA-1 protein as CD18 is also a subunit of Mac-1 (CD11b/CD18). It is, therefore, possible that Mac-1 is decreased and LFA-1 remains the same or is increased. The reason for the decrease in *paxillin* expression is equally obscure. Although paxillin has not previously been studied in corneal graft rejection, it is possible to postulate a role for this adhesion molecule in lymphocyte and/or macrophage infiltration of the cornea.[13–15] If this is the case, we must assume that cells with the highest levels of paxillin have entered the rejecting cornea leaving reduced levels in the peripheral blood. Unfortunately, it has not been possible to measure the levels of mRNA in rejecting human corneas and the infiltrating lymphocytes. The reasons for the decreased levels of

RhoA during corneal transplant rejection are obvious. However, it is known that *RhoA* and *Rac2* can be activated by two clearly distinct patterns, which are related to the adhesion of neutrophils to endothelial cells.[26] RhoA is also involved in the process of regulating antigen presentation in dendritic cells, which is important both for innate and adaptive immunity.[27]

Whether the differential expression of these genes enhance graft rejection, or graft rejection results in altered gene expression remains unclear. Future investigations should include further longitudinal studies analysing the expression of Rac2, paxillin, CD18, and RhoA at the protein level and determine which cells are responsible for their production. In our study, we were unable to take samples from rejecting human corneas for ethical reasons. However, future studies in animals are needed to confirm the role of these genes and their proteins in the rejection cornea. The role of glucocorticoids in the differential expression of the four genes in corneal transplantation patients with and without episodes of graft rejection should also be elucidated. In conclusion, our study demonstrated that the expression of *Rac2* was significantly upregulated in the peripheral blood of patients undergoing corneal transplantation who experience graft rejection, whereas the expression of *paxillin*, *RhoA*, and *CD18* were found to be significantly downregulated during corneal graft rejection. This may be of particular importance because of the involvement of these genes in T-cell differentiation, cytokine production, and lymphocyte adhesion.

Acknowledgements

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