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Gene expression profile during acute rejection in rat-to-mouse concordant cardiac xenograft by means of DNA microarray

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Abstract Using a rat-to-mouse concordant cardiac transplantation model and DNA microarrays, we studied the gene expression profiles during acute rejection. We used inbred BALB/c and C3H/He mice and Lewis rats for our study, in which heterotopic cardiac transplantations were performed. Total RNA was isolated from xenografts (Lewis to C3H), allografts (BALB/c to C3H), rat isografts (Lewis to Lewis) and mouse isografts (C3H to C3H) on day 5 following transplantation. We screened for gene expression profiles in the xenografts, allografts, and mouse isografts by means of DNA microarrays. With a murine array, we determined that many IFN-γ inducible genes were profoundly expressed in both the allografts and xenografts relative to the isografts. Mac-1 was specifically induced in the xenografts relative to the allografts. Using a rat array, we observed that the cardionatrin and atrial natriuretic factors were most profoundly expressed in the xenografts in comparison with the rat isografts. In addition to known genes, many expressed sequence tags were induced in the xenografts. We identified a group of genes, including Mac-1 induced specifically in xenografts, as well as many new genes upregulated in xenografts.

Keywords Transplantation · Delayed xenograft rejection · DNA microarray

Introduction

The enormous disparity between the supply of donor organs and the number of patients requiring transplantation for end-stage organ failure has resulted in research into the possibility of using donor organs from non-human sources.

In discordant models of xenotransplantation, vascularized grafts are rapidly rejected within a few minutes to a few hours after transplantation by a process of hyperacute rejection [9]. Significant progress in controlling hyperacute rejection has recently been achieved through the generation of transgenic pig donors expressing human complement regulators [5]. However, such xenografts are consistently rejected within 4–5 days thorough a process termed delayed xenograft rejection (DXR), which current immunosuppressive therapy has failed to overcome. DXR is characterized by mononuclear cell infiltration, endothelial activation, and thrombosis [1, 10]. However, to date, the exact mechanisms of DXR remain ill defined. The identity and abundance of mRNA species within a cell dictate, to a large extent, the potential biological activity of that cell. Cellular differentiation requires changes in patterns of gene transcription, as evidenced by the potent phenotypical alterations that result from the disruption of transcription factor genes. It is now possible to assess the mRNA profile of cells undergoing such changes globally, using recently developed DNA microarray technology [4, 15]. We recently identified the gene expression profile in acutely rejected murine cardiac allografts by means of a DNA microarray and indicated that IFN-γ signaling

played a pivotal role during acute allograft rejection [14]. Using this technique, we determined the gene expression profile in acutely rejected concordant cardiac xenografts in order to highlight the genes specifically involved in DXR. Our data indicate the usefulness of DNA microarray technology for this application and provide informative insights into the mechanism of DXR.

Material and methods

Mice

BALB/c H-2d and C3H/He H-2k mice (male, 6–8 weeks of age) and Lewis rats (8–15 days of age) were purchased from Clea Japan, (Tokyo, Japan). All mice and rats were kept in microisolator cages on a 12-h day/night cycle and fed on regular chow. All procedures involving experimental animals were carried out in accordance with protocols described in the local institutional guidelines for animal care at the University of Tokyo.

Heterotopic cardiac transplantation

Cardiac transplants were performed according to the method of Corry and co-workers. In brief, donors and recipients were anesthetized intraperitoneally prior to surgery with 4% chloral hydrate at 0.01 ml/g body weight. Donor hearts were perfused with chilled, heparinized saline via the inferior vena cava. The aorta and pulmonary artery of the donor hearts were anastomosed to the abdominal aorta and inferior vena cava of the recipients by a microsurgical technique. The viability of the cardiac allografts was assessed by abdominal palpation and confirmed by observation at laparotomy. Rejection of cardiac grafts was considered complete by the cessation of impulses and confirmed visually after laparotomy.

Histological examination

Cardiac grafts were removed from the recipients under anesthesia with 4% chloral hydrate on day 5 after transplantation. Each graft was cut transversely into two sections, and the basal portion was fixed in 8% paraformaldehyde, with the other section snap-frozen for RNA extraction. The section at the edge of maximal circumference was stained with hematoxylin and eosin.

RNA preparation

On day 5 after heterotopic cardiac transplantation, xenografts (Lewis to C3H/He), allografts (BALB/c to C3H/He) and isografts (C3H/He to C3H/He, Lewis to Lewis) were excised from the recipients. Total RNA was then isolated by Isogen (Nippon Gene) according to the manufacturer's protocol. The concentration of total RNA was determined by the optical density at 260 nm.

Microarray analysis

Intragraft gene expression in heterotopic heart grafts was examined via DNA microarray of isolated graft total RNA. The RNA was isolated from (a) rejecting cardiac xenografts (Lewis to C3H) on day 5 ($n=2$); (b) rejecting cardiac allografts (BALB/c to C3H) on day 5 ($n=2$); (c) mice cardiac isografts (C3H to C3H) on day 5 ($n=2$); (d) rat cardiac isografts (Lewis to Lewis) on day 5 ($n=1$). Total RNA was used to generate first-strand cDNA. After second-strand synthesis (Gibco BRL), in-vitro transcription (Ambion) was performed with biotinylated UTP and CTP (Enzo Diagnostics), resulting in 40–80-fold linear amplification of RNA. Amplified cRNA was purified on an affinity resin column (RNeasy, Qiagen) and quantitated by spectrophotometer. Forty micrograms of biotinylated RNA was fragmented to 50–150-nt fragments before overnight hybridization to Affymetrix (Santa Clara, Calif., USA) mouse 11K arrays (Mu11KsubA, Mu11KsubB) or rat U74A arrays. These arrays contain probe sets for more than 11,000 genes or 8,000 genes from mRNA transcripts or expressed sequence tag (EST) clones, respectively. The fragmented cRNA (to 0.05μg/μl), control oligonucleotide B2 (to 50 pM), control cRNA cocktail (to 5, 25, 100 pM), acetylated BSA (to 0.5 mg/ml) and sonicated herring sperm DNA (to 0.1 mg/ml) were added to the hybridization buffer containing 100 mM MES, 1.0 M NaCl, 20 mM EDTA and 0.01% Tween-20. The hybridization mixture was heated to 99 °C for 5 min, followed by incubation at 45 °C for 5 min before injection of the sample into the probe array cartridge. Hybridizations were carried out at 45 °C for 16–17 h while mixing on a rotisserie at 60 rpm. Following hybridization, the solutions were removed, the arrays were rinsed with non-stringent wash buffer (0.9 M NaCl, 51.9 mM $NaHPO_4$, 7.5 mM EDTA, 0.01% Tween-20 and 0.005% antifoam) for ten cycles of two mixes per cycle at 25 °C, and then incubated with stringent wash buffer (100 mM MES, 0.1 M NaCl, and 0.01% Tween-20) for four cycles of 15 mixes per cycle at 50 °C. Hybridized arrays were stained with 5.0 μg/ml streptavidin/phycoerythrin (Molecular Probes, Eugene, Ore., USA) and 2.0 mg/ml acetylated BSA (Gibco BRL) in SAPE solution (100 mM MES, 0.1 M NaCl, 0.05% Tween-20, 0.005% antifoam, 2 μg/μl acetylated BSA and 10 μg/ml SAPE) at 25 °C for 10 min. Following washes with non-stringent wash buffer, probe arrays were stained for 10 min at 25 °C in antibody solution (100 mM MES, 0.1 M NaCl, 0.05% Tween-20, 2 μg/μl acetylated BSA, 0.1 mg/ml normal goat IgG and 3 μg/ml biotinylated antibody). The probe arrays were then stained for 10 min in SAPE solution at 25 °C. The final wash entailed 15 cycles of four mixes per cycle at 30 °C with stringent wash buffer. Probe arrays were scanned three times at 3 μm resolution by the GeneChip system confocal scanner made for Affymetrix by Hewlett-Packard. Intensity values were scaled such that the overall intensity for each chip of the same type was equivalent [2].

Quantitative analysis

The intensity for each feature of the array was captured with GeneChip software (Affymetrix), and a single raw expression level for each gene was derived from the 20 probe pairs representing each gene by means of a trimmed mean algorithm. A threshold of 20 units was assigned to any gene with a calculated expression level below 20, because discrimination of expression below this level cannot be performed with confidence [7]. The expression level of each gene and the fold change between the two experiments was calculated by GeneChip software. Intensity values were scaled such that the overall intensity for each chip of the same type was equivalent. The average difference of each experiment was normalized to 100.

Results

Graft survival

In BALB/c to C3H/He cardiac allograft recipients, complete rejection occurred approximately 8 days after transplantation. In Lewis rat to C3H/He mouse cardiac xenografts, complete rejection occurred after approxi-

Table 1 Survival time of cardiac xenografts, allografts and isografts

Group (strain combinations)	Survival time (days)	Mean survival ± SD (days)
1. BALB/c to C3H/He	7, 8, 8, 8, 9	8 ± 0.6
2. Lewis to C3H/He	6, 7, 7, 7, 8	7.0 ± 0.7
3. C3H/He to C3H/He	> 100, > 100, > 100	> 100*
4. Lewis to Lewis	> 100, > 100, > 100	> 100*

*$P < 0.05$, compared with groups 1 or 2

mately 7 days. Cardiac isografts in both C3H mice and Lewis rat functioned for more than 100 days (Table 1).

Histological assessment

On day 5, there was no difference in terms of cellular infiltration between the allografts and xenografts, both of which showed diffuse, perivascular or interstitial infiltration of mononuclear cells and some foci of inflammatory infiltration with myocyte damage on day 5. None of the isografts in any of the groups underwent rejection.

DNA microarray

Since each sample was hybridized to a separate DNA array, it was essential we determine the consistency of the arrays by calculating the average intensities for all of the GAPDH probes in all the data sets. We observed that the average hybridization signals for each GAPDH probe set differed by less than 50% in all of the samples. We screened for gene-expression changes in the allograft using Affymetrix oligonucleotide expression arrays to monitor tens of thousands of genes and ESTs.

First, we analyzed genes profoundly induced in the xenografts on day 5 by means of a DNA chip for mice. The threshold values of an average difference of at least 100 and a fold-change of at least 3.0-fold were considered reliable for genes with expression values significantly over the background values. A total of 346 genes was expressed in all rejecting cardiac xenografts on day 5. The top 20 genes induced most profoundly in the xenograft on day 5 are shown in Table 2. Many IFN-γ inducible genes, including CXC chemokine Mig (monokine induced by IFN-γ), were upregulated in both allografts and xenografts. Interestingly, serine proteinase inhibitor mBM2A, c-fos oncogene and cell surface glycoprotein Mac-1 were specifically induced in cardiac xenografts. We next analyzed the gene expression in xenografts and rat isografts by means of a DNA chip for rats. The top 20 genes induced most profoundly in the xenograft on day 5 are shown in Table 3. The cardionatrin gene was most profoundly induced, followed by atrial natriuretic factor (ANF). Several ESTs with an unknown function, whose mechanism in and relationship to graft rejection are unknown, were upregulated in the cardiac xenografts. The gene expression of Fas antigen was profoundly induced in the xenograft by means of a DNA chip for rats (fold change, 15.3), while not induced by means of a DNA chip for mice (fold change, 1.6). These data suggests apoptotic responses in the transplanted hearts.

Discussion

We examined gene expression profiles in cases of acute rejection in rat-to-mouse cardiac xenografts and identified many genes involved in rejection. A group of genes, including Mac-1, were upregulated specifically in concordant xenografts. The precise pathogenesis of DXR is largely unknown. Conventional therapy, which is effective in blocking allograft rejection, failed to overcome the DXR. T cells play an important role in DXR as well as in allograft rejection [13], and macrophages or humoral immunity are also reported to be pivotal [3, 16, 17]. Our data provide gene expression profiles during acute rejection in cardiac xenografts and allografts on a genome-wide scale.

Recently, we reported that IFN-γ signaling is important in murine cardiac allograft rejection, as determined by means of DNA microarray. IFN-γ-inducible genes were also profoundly induced in cardiac xenografts. Furthermore, we identified a group of genes induced specifically in cardiac xenografts, including Mac-1, c-fos and serine proteinase-1.

Mac-1 is a CD11b/18 cell membrane glycoprotein that is an important adhesion molecule involved in the migration of leukocytes and expressed by monocytes. Mac-1, as well as their counter-receptor, intercellular adhesion molecule-1 (ICAM-1), plays an important role in transendothelial migration [6]. Serine proteinase inhibitor (SERP-1) has been shown to interact with the thrombolytic cascade, and thereby reduce proteolysis, chemotaxis and adhesion [11]. In a balloon-injury model of rabbit aorta, a single dose of SERP-1 significantly decreased the amount of early (24-h) mononuclear cell infiltration and late intima hyperplasia 30 days post-injury [12]. These reports indicate that the SERP-1 gene expression does not have a deleterious effect on DXR but does ameliorate DXR. Recently, SERP-1 was reported to be a protective gene for acute rejection in

Table 2 Top 20 genes profoundly induced in rat-to-mouse cardiac xenograft using mouse arrays. The top 20 most profoundly induced genes in cardiac xenografts vs. isografts on day 5; 346 genes were induced in every rejecting cardiac allograft on day 5. These genes were sorted by fold-change in the xenograft in the first experiment vs. the isograft in the first experiment day 5, and the top 20 genes were listed. *1st* first experiment, *2nd* second experiment

Rank	Accession no.	Gene description	Average difference							Fold change
			Murine isograft (1st)	Murine isograft (2nd)	Rat isograft	Allograft (1st)	Allograft (2nd)	Xenograft (1st)	Xenograft (2nd)	Xenograft vs. isograft (1st)
1	x62742	*M. musculus* Ma mRNA	−110	−365	−403	480	199	723	886.8	180.5
2	d14566	Lmp-2 gene for LMP-2 polypeptide (complete cds)	−44	−91	−51	836	828	1041	1189	151.2
3	M34815	Monokine induced by gamma interferon (Mig)	−4	−6	−56	1402	1133	895	13605	1267
4		Signal transducer and activator of transcription (Stat1)	−25	−172	−67	326	340	532	644	1187
5	U96701	Serine proteinase inhibitor mBM2A	−32	−73	48	−54	−25	370	220.8	101.3
6		Immune-responsive gene 1 (Irg1)	−3	35	−24	227	195	404	459.2	80.6
7	ab000677	JAB	−42	−33	29	510	310	544	398	75.4
8	L38281	Immune-responsive gene 1 (Irg1)	7	11	33	401	385	599	647.3	72.8
9	v00727	c-fos oncogene	3	−56	−20	76	45	313	196.4	72.7
10	X07640	Cell surface glycoprotein Mac-1 alpha-chain	0	−13	17	18	49	260	203.6	69.8
11		Class II histocompatibility antigen, M alpha chain	0	−300	59	15	−95	239	164.6	59.7
12	U53219	GTPase IGTP	4	54	−85	1366	1768	495	598.5	59.4
13		Argininosuccinate synthetase (Ass)	5	−269	−122	360	153	283	380.5	59.2
14	x02339	T3 delta chain of T3/T-cell receptor glycoprotein	−53	26	−23	42	209	158	258.1	56.2
15	aa415898	EST	33	275	7	1092	1628	1748	1717	44.6
16	D44464	Uridine phosphorylase	23	22	35	207	174	313	526.8	43.9
17		*M. musculus* vav mRNA	22	−31	−127	100	69	187	208	43.4
18		Interferon-gamma inducible protein Mag-1	16	32	−2	845	831	583	568.1	43.3
19		Interferon-inducible protein 9–27	15	10	−41	106	85	166	146.7	42.5
20		Interferon-activatable protein 204 (IFI-204)	4	22	−27	126	124	174	243.3	40.3

Table 3 Genes induced profoundly in rat-to-mouse cardiac xenograft using rat arrays. The top 20 genes induced most profoundly in the xenograft on day 5 are shown

Rank	Accession no.	Gene description	Average difference		Fold change
			Xenograft (rat-to-mouse)	Isograft (rat-to-rat)	Xenograft vs. Isograft
1	E00775	Rat cardionatrin precursor	1595	−10	392.6
2	E00903	Rat ANF	1076	−50	265.3
3	AA892553	EST	826	−15	180.4
4	AI102031	EST	337	−73	95
5	AI639117	EST	344	−151	94.4
6	M80367	Rat isoprenylated 67-kDa protein	644	7	70.8
7	U17035	*Rattus* sp. (mob-1)	1185	17	70.1
8	M64795	Rat MHC class I antigen gene (RT1-u haplotype)	255	−24	65.1
9	AB010119	*R. norvegicus* mRNA for Tctex-1	256	11	62.3
10	M34253	Interferon regulatory factor 1 (IRF-1)	232	7	59.8
11	AA800602	EST	155	−84	55.8
12	U31599	MHC class II-like beta chain (RT1.DMb)	180	−20	52.2
13	X61479	RRCSF1 rat mRNA for CSF-1 receptor	540	10	50
14	U17565	*R. norvegicus* intestinal DNA replication protein	205	4	46.5
15	L42293	Acyl-coenzyme A:cholesterol acyltransferase (ACACT)	207	4	42.3
16	M84488	Vascular cell adhesion molecule-1	196	2	39.3
17	Z14030	TRAP-complex gamma subunit	215	3	35.9
18	AA859896	EST	164	−5	34.5
19	U65217	*R. norvegicus* MHC class II antigen	109	−2	34.2
20	E00593	Polypeptide having immunoglobulin E binding factor activity	117	−21	32.8

cardiac transplants [8]. It is reasonable to consider the SERP-1 gene expression as a protective reaction against rejection. We also analyzed gene expression profiles by means of rat-GeneChip and found that ANF was most profoundly induced. Vascular cell adhesion molecule-1 (VCAM-1) and interferon regulatory factor-1 (IRF-1) were also upregulated. These genes were considered to be expressed in the donor tissue. Therefore, the genetic alteration of these genes in the donor organ may be a potential therapeutic target for avoiding DXR. Mice and rats have much genomic homology; therefore, we analyzed rat genes with mouse-GeneChip. We compared gene expressions in murine cardiac isografts and rat cardiac isografts by means of mouse-GeneChip. Because the same physiological stimulation, i.e., ischemic-reperfusion injury, is thought to alter the following genetic transcription in isografts in both mice and rats, we hypothesized that the genes profoundly induced in both cardiac isografts were common. We analyzed the data of the genes profoundly induced in cardiac isografts and found that some of these gene expressions were also detected in rat cardiac xenografts by means of mouse-GeneChip (data not shown). These results indicate that mouse-GeneChip can detect gene expressions of some rat genes as well as mouse genes. In conclusion, our results reveal a molecular profile in rat-to-mouse cardiac xenografts that allowed us to identify a group of genes, including Mac-1, induced specifically in the xenograft, as well as many new genes upregulated in xenografts. Since the rat-to-mouse xenograft model is highly dependent on T-cell immunity, the relationship between DXR and the genes profiled in this study should be clarified in various DXR models. In this study, DNA microarrays have provided useful and important information that will enable further study of DXR.

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