Identification of gene expression profile in tolerizing murine cardiac allograft by costimulatory blockade

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Matsui, Yuichi, Akio Saiura, Yasuhiro Sugawara, Masataka Sata, Katsutoshi Naruse, Hideo Yagita, Takahide Kohro, Chikage Matakii, Akashi Izumi, Takahiro Yamaguchi, Takashi Minami, Toshiko Sakihama, Sigeo Ihara, Hiroyuki Aburatani, Takao Hamakubo, Tatsuhiko Kodama, and Masatoshi Makuuchi. Identification of gene expression profile in tolerizing murine cardiac allograft by costimulatory blockade. Physiol Genomics 15: 199–208, 2003. First published September 9, 2003; 10.1152/physiolgenomics.00086.2003.—The induction of specific tolerance would be the ultimate achievement in transplant immunology, but the precise mechanisms of immunologic tolerance remain largely unknown. Here, we investigated global gene expression analysis in tolerizing murine cardiac allografts by means of oligonucleotide microarrays. Tolerance induction was achieved in cardiac allografts from BALB/c to C57BL/6 mice by daily intraperitoneal injection of anti-CD80 and anti-CD86 monoclonal antibodies (mAbs). Comparative analysis revealed 64 genes to be induced more extensively in the tolerizing than in the syngeneic isografts, and 16 genes than in the rejecting allografts. Two genes were specifically upregulated in the tolerizing allografts. In the tolerizing allografts there were induced marked expressions of a number of genes for pro-inflammatory factors, including interferon-γ-inducible cytokines and chemokines, as well as apoptosis-related genes, which were also upregulated in the rejecting allografts. Moreover, these gene expression patterns continued to be upregulated more than 70 days posttransplant. These results provide evidence that immunologic tolerance can be induced and maintained in the presence of prominent pro-inflammatory gene expression in vivo. transplanted; immunologic tolerance; interferon-γ; chemokine; DNA microarray

Although recent advances in immunosuppressive therapy have dramatically enhanced the early survival of cardiac transplant recipients, acute rejection still occurs in ~50% of the recipients (21). Long-term immunosuppressive drug administration, furthermore, entails a number of potentially significant problems such as infection, spontaneous neoplasm, undesirable metabolic effects and drug toxicity. Alloantigen-specific tolerance induction is the ultimate goal in transplant immunology, and can be induced in a rodent model; however, the precise mechanisms by which specific tolerance is affected are not clearly understood, and the current immunosuppression regimens have all failed to achieve this goal in a clinical setting.

The mechanisms of allograft tolerance have been classified into categories of central and peripheral tolerance (14). Peripheral tolerance can be induced by the blockade of the T cell costimulatory pathway (11). Recent studies have demonstrated that activation-induced cell death of T cells is required for tolerance induction (22). Interleukin-2 (IL-2) and interferon-γ (IFN-γ) promote activation-induced cell death (13, 22). Consequently, IL-2-deficient and/or IFN-γ-deficient mice are resistant to tolerance induction (22). However, although there are increasing reported data describing the molecular mechanisms of tolerance induction, no systematic analysis has been carried out.

DNA microarray technology has made it possible to analyze the expression of a large number of genes and revolutionized many areas of biology and medicine (4). This new technology can provide unbiased, global expressions of tens of thousands of genes simultaneously. Recent studies on gene expression profiles in various cardiac diseases, including cardiac allograft rejection, have successfully provided important information and new insights into the biological mechanisms of these diseases (23). The precise molecular mechanism of immunologic tolerance, however, has been relatively poorly dissected by comparison.

Here we provide a gene expression profile of the tolerizing allografts after costimulatory signal blockade in a murine cardiac transplant model. We also demonstrate vigorous gene expressions of pro-inflam-

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matory cytokines and chemokines, apoptosis, and signal transduction in the tolerizing cardiac allografts in vivo.

METHODS

Mice. Male BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from Clea Japan (Tokyo, Japan). Adult males 6–8 wk of age were used throughout the study. All mice were kept in microisolator cages on a 12:12-h day/night cycle and fed on regular chow. All procedures involving experimental animals were carried out in accordance with protocols approved in the local institutional guideline for animal care of The University of Tokyo and complied with the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 86-23, revised 1985).

Monoclonal antibodies. Hybridomas producing anti-B7-1 and anti-B7-2 monoclonal antibodies (mAbs 1G10 and GL1, respectively) were kind gifts from Dr. Yagita of Juntendo University.

Heterotopic cardiac transplant. Cardiac transplants were performed according to the method of Corry and coworkers (6). In brief, donors and recipients were anesthetized intraperitoneally prior to surgery with 4% chloral hydrate at 0.01 ml/g body wt. 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 means of a microsurgical technique. The viability of the cardiac allografts was assessed by daily transabdominal palpation and confirmed by observation at laparotomy. Rejection of cardiac grafts was considered complete by the cessation of impulses and confirmed visually after laparotomy. Some recipients were injected intraperitoneally with 100 μg each of anti-CD80 and anti-CD86 mAbs daily for 5 days after transplantation (2).

Histological examination. Heart allografts and isografts were removed from the recipients under anesthesia with 4% chloral hydrate on day 7 posttransplant. The graft was cut transversely into two sections, and the basal portion was fixed in 4% paraformaldehyde with the other section snap frozen for RNA extraction. The section at the edge of the maximal circumference was stained with hematoxylin and eosin.

Oligonucleotide microarray. After heterotopic cardiac transplantation, transplanted hearts were excised from the recipients and snap frozen in liquid nitrogen and stored at −80°C until use. Total RNA was then isolated independently using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol. The quality of extracted total RNA was confirmed with an Agilent 2100 Bioanalyzer (Palo Alto, CA) or electrophoresis on 1.5% agarose gels stained by SYBR Green (Applied Biosystems, Foster City, CA).

Double-stranded complementary DNA was synthesized from 10 μg of total RNA according to Affymetrix (Affymetrix, Santa Clara, CA) methodology, and cDNA was purified with Phase Lock Gels (Eppendorf, Hamburg, Germany). We synthesized biotin-labeled RNA with the BioArray High Yield RNA Transcript Labeling Kit (Enzo, New York, NY).

Hybridization from biotinylated cRNA to murine genome GeneChips (MG-U74Av2, Affymetrix) was performed in accordance with the manufacturer’s instructions, stored at 40°C overnight, heating in a mix that included 10 μg fragmented RNA, 6X SSPE, 0.005% Triton X-100, and 100 mg/ml herring sperm DNA in a total volume of 200 μl.

GeneChips were washed and stained with streptavidin-c (Molecular Probes, Eugene, OR), and probe arrays were scanned three times at 3-μm resolution using the GeneChip system confocal scanner made for Affymetrix by Hewlett-Packard.

MicroArray Suite (MAS) version 5.1 (Affymetrix) was utilized to calculate from the scanned images 1) average difference, 2) log ratio (base 2), and 3) absolute call. Intensity values were scaled such that the overall intensity for each GeneChip of the same type was equivalent (4). The average difference of each experiment was normalized to 100 to allow comparison among multiple arrays.

To determine the absolute call (detected or not detected) of a specific probe set, the number of instances in which the perfect match (PM) hybridization signal was greater than the mismatch (MM) signal was computed by MAS ver. 5.1 (Affymetrix), along with the average of the logarithm of the PM/MM ratios for each probe set.

GEO accession numbers. Array data were deposited at the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information with accession numbers GSM8926 through GSM8939. The array data are also available in series with accession number GSE5582.

Data analysis. The gene expression profile consisted of the following three groups: group A, syngeneic isograft (C57BL/6 to C57BL/6); group B, rejecting allograft (BALB/c to C57BL/6); and group C, tolerizing allograft (BALB/c to C57BL/6 with daily intraperitoneal injection of anti-CD80 and anti-CD86 mAbs for 5 days after transplantation). Time course data was also collected on day 7 (n = 1), day 7 (n = 3), day 28 (n = 2, group C) and day 70 (n = 2, group A; n = 3, group C) after transplantation.

We compared the microarray data derived from the nine samples of the transplanted hearts excised on day 7. Analyses were performed based on the average difference, log ratio (base 2), and absolute call (presence or absence) for each gene among the triplicate samples of each group (Fig. 1). First, to exclude obscuring noise, all 12,488 probes on the MG-U74Av2 array were filtered by the average difference and absolute call (present or absent). The threshold values of an average difference of more than 50 in all three samples of the objective group, and at least one present call from among three in the group, were considered reliable for genes with expression values significantly over background. Second, we compared the log ratios (base 2), calculated by MAS 5.1 (Affymetrix). The threshold values of log ratio (base 2) of more than 1.58 (nearly equal to 3.0-fold) were considered reliable for genes upregulated significantly over baseline expression. Finally, singled-out genes were analyzed statistically with one-sided t-test and adjusted by means of a permutation-style resampling method using SAS version 8.2 (SAS/MULTTEST, SAS Institute) with significance set at P ≤ 0.05 to control for the false-positive error rate associated with multiple tests. As for those genes screened out by these analyses, the time course data on days 5, 28, and 70 after transplantation were also compared.

We used the program GenoSpring version 4.0 (Silicon Genetics, Redwood City, CA) to construct hierarchical clustering. Similarity was measured by standard correlation of the average difference for each gene, which was normalized to itself by making a synthetic positive control for that gene and dividing all measurements for that gene by this positive control, assuming it was at least 1.0. This synthetic control was the median of the gene’s expression values over all the samples. Data are presented in a matrix format. Each row represents a single gene, and each column an experimental sample. The ratio of the abundance of transcripts of each gene to the median abundance of the gene’s transcript is represented by a color in the corresponding sample in the matrix. Green squares indicate transcript
levels below the median; red squares indicate transcripts levels above the median; and white shadow signifies a lack of trust in the data.

Quantitative RT-PCR. To confirm the microarray data, quantitative real-time PCR was performed in an iCycler (Bio-Rad, Hercules, CA) using a SYBR Green PCR kit from Applied Biosystems and specific primers to amplify 100-200-bp fragments from the different genes analyzed. A threshold was set in the linear part of the amplification curve, and the number of cycles needed to reach the threshold was calculated for every gene. Normalization was achieved by including a sample with primers for hypoxanthine guanine phosphoribosyl transferase (HPRT) as a control.

To synthesize single-stranded cDNA, 1/10 μg of total RNA isolated from transplanted hearts on days 7 and 70 (n = 6, respectively; distinct from the samples used for microarray analysis) was reverse transcribed using SuperScript II reverse transcriptase and an oligo(dT)$_{12-18}$ primer (Invitrogen Japan, Tokyo, Japan) in a final volume of 20 μl and diluted up to 80 μl.

PCR was then performed with 1 μl of cDNA for 1 cycle of 94°C for 3 min, followed by 35–40 cycles of 94°C for 15 s, 60–68°C for 15 s, and 72°C for 30 s. The primer sets used are listed in Table 1. PCR products were separated by electrophoresis on 1.5% agarose gels and were visualized with ethidium bromide staining.

RESULTS

Graft survival. All the isografts and allografts in mice administered monoclonal antibody against CD80 and CD86 from on days 0 to 4 survived more than 100 days (n = 5, respectively). Mean graft survival time for
cardiac allografts without administration of mAb \( n = 5 \) was \( 8 \pm 0.7 \) days (mean ± SD).

**Histological findings.** On day 7, tolerizing allografts exhibited a slightly diffuse, perivascular, or interstitial infiltration of mononuclear cells. Diffuse, perivascular, or interstitial infiltration of mononuclear cells and some foci of inflammatory infiltration with myocyte damage were observed in rejecting allografts. In addition, none of the isografts underwent rejection, as would be expected for inbred mouse strains (Fig. 2).

**Reproducibility of the data.** To verify the reproducibility of the data, the correlation among triplicate samples excised on day 7 was investigated. We confirmed a strong linear correlation \( r > 0.95; r \) is Pearson’s correlation coefficient) between the gene expression profiles in the replicate samples of the same group (Fig. 3, A–C). On the other hand, there are larger alterations \( 0.7 < r < 0.9 \) in expression pattern between the genes of the isografts, rejecting allografts, and tolerizing allografts (Fig. 3, D–F).

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**Table 1. Primer pairs used in quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sense (5’ → 3’)</th>
<th>Antisense (3’ → 5’)</th>
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<tr>
<td>HPRT</td>
<td>K01515</td>
<td>TGAAGAGCTACTGTAATGATCAGTCAA</td>
<td>AGCAAAGCTTGCAAACCTTAACA</td>
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<tr>
<td>MIG</td>
<td>M34815</td>
<td>ATTTTCATCGGCGCTTTGAGGAGGCTT</td>
<td>AAAGGACGAGCGCTGAGATTTG</td>
</tr>
<tr>
<td>RANTES</td>
<td>AF065947</td>
<td>GCTGCCCTCAGCCTCCCTTCAACA</td>
<td>TTCTCTGGTGGGGACAGACCTT</td>
</tr>
<tr>
<td>H2-Ea</td>
<td>V00833</td>
<td>GAAATCCTGGTTGCTCTTGTTGTT</td>
<td>TGAAGCATTGGCGCTCAAGAT</td>
</tr>
<tr>
<td>FRZB</td>
<td>U88058</td>
<td>CAAAGAGGTAGGCGACAGCCCAAAC</td>
<td>TGATTCCAGTGGTGGGGTAA</td>
</tr>
<tr>
<td>Gip1</td>
<td>M55544</td>
<td>ATCTTGGGGAGGGTGTGCTTTTCA</td>
<td>GAAAGGAAACAGCTGAGGAG</td>
</tr>
<tr>
<td>Itgb2</td>
<td>M31039</td>
<td>AGCAGAAGAGGGAGGAAGGACAT</td>
<td>ACCAATGAGAGGAGGAG</td>
</tr>
<tr>
<td>Plaur</td>
<td>X62700</td>
<td>CTATGGGGCGTCTCTCTCGTC</td>
<td>GGAGCCACAGATCGTCAAG</td>
</tr>
<tr>
<td>TGFb</td>
<td>L19932</td>
<td>AAGGATGTAAGGGAGGAACAC</td>
<td>TGATGGCTCAGAAGAGACAG</td>
</tr>
<tr>
<td>IL1b</td>
<td>M15131</td>
<td>ACATAGGACAGTGAACAGGAGCA</td>
<td>AGGAAAGTGCTGAGGAG</td>
</tr>
<tr>
<td>F1n1</td>
<td>M18194</td>
<td>TTAGGAGAAGAAGTCCACCAAAAC</td>
<td>TTAGGAGAAGAAGTTGGAG</td>
</tr>
<tr>
<td>KRAB</td>
<td>AB024905</td>
<td>TACTTCTCAGAGATCGAGCAAA</td>
<td>GTTTGCTAGAACAGGAGG</td>
</tr>
<tr>
<td>NFATc1</td>
<td>AF087434</td>
<td>TTGGTAAAGAGAGGAGGTCGC</td>
<td>ATACGCTACGACAGGAG</td>
</tr>
<tr>
<td>Vav1</td>
<td>X64361</td>
<td>AAGGAGGCTCCTCCTCTCTTAA</td>
<td>ATACGTAGAGGAGGAG</td>
</tr>
<tr>
<td>Casp4</td>
<td>Y13089</td>
<td>TCTGGAAGCTAGGAGAAGAAGG</td>
<td>GTTGCTTAGAGGAGGAG</td>
</tr>
<tr>
<td>Dcn</td>
<td>X53929</td>
<td>GATGGTTATGAGGATGACTT</td>
<td>GGGGTGTGTTCCAGATTAT</td>
</tr>
<tr>
<td>Cox6a2</td>
<td>U08439</td>
<td>ACCAAGCTTTTCAGGAAAGAG</td>
<td>ACTTCGACACCTTTTATTGAG</td>
</tr>
</tbody>
</table>

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**Fig. 2. Histology of transplanted hearts (A–C).** On day 7, transplanted cardiac grafts were harvested and processed for hematoxylin and eosin staining. A: tolerizing allograft treated with mAbs 1G10 and GL1 from days 0 to 4 daily. B: rejecting allograft. C: syngeneic isograft. Bar = 50 μm.
Genes induced in tolerizing cardiac allografts. The analyses described in the METHODS revealed that 64 genes or expressed sequences tags (ESTs) were expressed to a significantly greater extent (P < 0.05) in the tolerizing allografts than in the syngeneic isografts on day 7 (Fig. 1A; also, see Supplementary Table 1, available at the Physiological Genomics web site). The same analyses performed in tolerizing vs. rejecting allografts elicited 16 genes to be induced more extensively in the tolerizing than rejecting allografts (Fig. 1A). The expression patterns of the former 64 genes were clustered by means of GeneSpring (Fig. 4A). Representative chemokine gene expression is shown in Fig. 4B. The average difference of not only the tolerizing but also the rejecting cardiac allografts on day 7 was three times more than that of the syngeneic isografts on day 7. Fifty-six of these 64 genes were also induced more extensively in the rejecting allografts than syngeneic isografts. Genes identified commonly in both analyses of tolerizing allografts vs. syngeneic isografts and tolerizing vs. rejecting allografts were considered to be genes upregulated specifically in the tolerizing allografts (Fig. 1A). Two genes, histocompatibility 2, class II antigen Ea (H2-Ea) and secreted frizzled-related protein (FRZB) were identified, and the average difference and log ratio of these two genes are listed in Table 2. The expression pattern of H2-Ea is shown in Fig. 4C.

Genes induced in rejecting cardiac allografts. Next, we analyzed the gene expression induced in rejecting cardiac allografts. We had previously identified 84 genes or ESTs induced in rejecting cardiac allografts and had shown that IFN-γ-inducible genes are prominently induced in cardiac allografts (20). In this list of 84 genes, 66 probe sets were available in the setting of these experiments. Differential expression patterns of these 66 genes in isografts on days 5, 7, and 70, rejecting allografts on days 5 and 7, and tolerizing allografts on days 5, 7, 28, and 70 are shown in Fig. 5A. Interestingly, all of the preponderance of the genes profoundly induced in the rejecting allografts were also induced in the tolerizing cardiac allografts. We focused on chemotactic pro-inflammatory cytokine genes, especially on MIG and RANTES, because of the close relationship to the involvement in acute rejection (10, 20). The expression pattern of these two genes is shown in Fig. 4B. MIG and RANTES were also upregulated significantly (P < 0.05) in the tolerizing cardiac allografts. Furthermore, these gene expressions continued to be upregulated even on the 70th day after transplantation, more extensively in the tolerizing cardiac allografts than in the nonrejecting isografts.

Genes induced specifically in rejecting cardiac allografts. Many genes were upregulated both in the rejecting and tolerizing cardiac allografts. Therefore, we focused on the differential expression pattern between the two so as to identify a group of genes upregulated specifically in rejecting cardiac allografts. The criteria were the same as those performed to identify genes induced specifically in the tolerizing allografts, and the algorithm is shown in Fig. 1B. The analyses revealed that the expression of 21 genes was upregulated specifically in the rejecting allografts (Supplementary Ta-
A clustering analysis of these 21 genes is shown in Fig. 5B. A clustering analysis of these 21 genes is shown in Fig. 5B.

Gene expressions in functional families. To characterize gene expression, we grouped the genes according to their functions (Fig. 6 and Supplementary Table 3). Hierarchical clustering revealed the expression of a large number of pro-inflammatory cytokines and chemokines was upregulated in both the rejecting and tolerizing allografts on day 7. Tol, tolerizing cardiac allografts; Iso, syngeneic cardiac isografts; Rej, rejecting cardiac allografts.

Table 2. Genes upregulated specifically in the tolerizing allograft on day 7

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>Day 7</th>
<th>Day 70</th>
<th>Day 7 Log Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>V00833</td>
<td>H2-Ea</td>
<td>E-alpha gene for immune response gene</td>
<td>4.8 ± 2.9</td>
<td>105.3 ± 97.0</td>
<td>467.5 ± 75.1</td>
</tr>
<tr>
<td>U68058</td>
<td>Frzb</td>
<td>frizzled-related protein</td>
<td>29.8 ± 20.4</td>
<td>4.8 ± 1.3</td>
<td>101.9 ± 14.4</td>
</tr>
</tbody>
</table>

Values are average differences ± SD and log ratios of the 2 genes, frizzled-related protein, and H2-Ea, induced specifically in the cardiac tolerizing allografts on day 7. Tol, tolerizing cardiac allografts; Iso, syngeneic cardiac isografts; Rej, rejecting cardiac allografts.
tolerizing allografts. MIG, RANTES, IP-10, and MCP-2 and their receptors CCR1, CCR5, and CXCR3 were induced not only in the rejecting but also in the tolerizing allografts on day 7 (Fig. 6A).

In a group of adhesion molecule, signal transduction, and apoptosis-related genes, most of the expression patterns were found to be prominent not only in the rejecting but also in the tolerizing allografts. In contrast, we were able to identify a group of genes that was downregulated exclusively in the rejecting allografts in a group of genes related to cell structure/motility and cell metabolism (Fig. 6B).

**Validation of gene expression data by quantitative RT-PCR.** To further validate the microarray data on changes in gene expression, certain representative genes upregulated specifically in the rejecting and tolerizing allografts, as well as genes of their functional families, were analyzed by quantitative RT-PCR (Fig. 7). The microarray data expression patterns were verified for the 16 genes except Fn1, KRAB, FRZB, and Casp4. In particular, the expression patterns of three of the genes (Itgb2, Gbp1, and Dcn) were found to have a perfect match between their quantitative RT-PCR and DNA microarray data in terms of patterns of statistical significance.

Compared with the deteriorated expression in the isografts on days 7 and 70, the expression of MIG and RANTES continued to be upregulated in both groups of the rejecting and tolerizing allografts, which correlated well with the data from the DNA microarray.

**DISCUSSION**

We describe the gene expression profile of tolerizing cardiac allografts compared with syngeneic isografts and rejecting allografts. It proved possible to identify the prominent gene expression of many putative pro-inflammatory cytokines and chemokines in tolerogenic allografts. Our results indicate that these pro-inflammatory cytokines and chemokines are closely associ-
ated with tolerance induction by costimulatory blockade.

In this study, we demonstrated the similarity of gene expression profile in the states of acute rejection and tolerance. No effective regimen has succeeded in inducing tolerance in clinical organ transplantation (19). Our findings point to an association between the induction of tolerance and many functional signals including inflammation, apoptosis, and signal transduction. It is interesting that marked expression of genes for various kinds of pro-inflammatory cytokines, chemokines, and adhesion molecules did not break the tolerance state. Moreover, these genes continued to be upregulated even 70 days after transplantation. These findings suggest that these genes have no deleterious effects on the tolerance state not only in the induction phase but also in the maintenance phase.

Recent reports support the idea that calcineurin-NFAT signaling is absolutely required in the induction of tolerance both in vitro and in vivo (17). Although the current immunosuppressive regimens have dramatically improved early survival after transplantation, they have failed to induce tolerance. Paradoxically, tolerance induction by costimulatory blockade is hindered by calcineurin inhibitors and steroids (12, 15), which are commonly employed in the standard regimen of immunosuppressant drugs after organ transplant. This may be one of the reasons for the failure to date of all attempts to induce tolerance after organ transplantation. Another studies have demonstrated a
requirement for activation-induced cell death of T cells in tolerance induction (13). IL-2 and IFN-γ promote activation-induced cell death (1, 3, 7). These are consistent with our results demonstrating prominent expressions of apoptosis-related genes. Our data revealed prominent IFN-γ signaling both in rejecting and tolerizing cardiac allografts. Taken together, it may be that IFN-γ signaling contributes to tolerance induction by promoting the activation-induced cell death of T cells. If so, costimulatory blockade together with immunosuppressive drugs which do not block the signaling of IFN-γ or IL-2, such as mycophenolate mofetil, sirolimus, or IL-2 receptor antibodies, would be more feasible in the induction of tolerance after clinical transplantation.

In contrast to isografts, moderate mononuclear cell infiltrations were observed in the tolerizing cardiac allografts on day 7. Recruitment of mononuclear cells into the graft appears to be an essential step for tolerance induction. Pro-inflammatory cytokines or chemokines may play a pivotal role in this step by helping to recruit these mononuclear cells. It is interesting that despite mononuclear cell infiltrations indefinite graft survival can be achieved in the tolerizing allografts. A group of genes related to cell structure and motility were exclusively suppressed in the rejecting allografts. Consistent with the macroscopic viability and indefinite graft survival, these data indicate the viability of the graft. Some as yet unknown mechanism which inactivates the infiltrated cells ability to attack the graft protects the tolerizing graft from rejection.

Recently, AP-1 signaling was selectively suppressed in tolerizing allografts compared with rejecting allografts (17). We analyzed 14 of the 17 genes reported in that study. The 14 genes were not altered in either the tolerizing or rejecting allografts in our study. Further study will be necessary to identify how the difference in gene expression between the rejecting and tolerizing allografts actually determines the different outcomes, as well as to clarify the molecular mechanism of immunologic tolerance.

Although DNA microarray analysis had previously shown the similarity of gene expression in cardiac rejecting and tolerizing allografts, we were able to identify only a few genes, e.g., H2-Ea and secreted frizzled-related protein (FRZB), as specifically induced in the tolerizing cardiac allografts. H2-Ea in the mouse is a homologous gene of human lymphocyte antigen (HLA) DR-α in humans, and in clinical renal transplantation HLA-DR matching of cadaveric kidneys has been shown to improve survival of the graft (18). FRZB is an antagonist of Wnt signaling, which has an important role in the differentiation and patterning of tissues in animal development (8). We could not identify the mechanism by which the induced gene expressions of H2-Ea and FRZB in the tolerizing cardiac allografts contribute to the induction and maintenance of tolerance. It is expected that future work will elucidate both, and ultimately afford us an insight into how tolerance can be manipulated in the clinical setting.
DNA microarray technology is a powerful tool in an unbiased assessment of expression levels of thousands of genes simultaneously, but there are several issues which must be kept in mind when interpreting the results this technology yields (9, 16). First, most studies are performed with a rather more limited number of repetitions than might be optimally desirable, because of the high cost of microarray studies, and reducing the number of replications could conceivably lead to false-positive results. Another point to keep in mind is that the validity of the data often depends on the experimental settings and the investigator’s technical skills. Our previous investigations led to reports of a good correlation of the data obtained by DNA microarray and Northern blotting. We demonstrated a reliable reproducibility of the data between replicate experiments. Second, the correlation between the mRNA and the target protein relative abundance in the cell may not be straightforward. This issue is undoubtedly problematic, but the actual absence of mRNA in the cell is certainly likely to imply a not very high level of the respective protein (5). Therefore, our current data with a set of replicate samples can provide us adequate information. Finally, it is difficult to detect a weak gene expression signal by means of DNA microarray technology. Such diminished but yet still biologically important gene expression could well be masked by other vigorous gene expressions.

In this study, we have reported the gene expression profile of tolerizing allografts in a murine cardiac transplant model. Our findings indicate that immunological tolerance can be induced and maintained in the face of marked pro-inflammatory gene expression in vivo. The DNA microarray has provided us with new insight into the molecular mechanisms of immunological tolerance after costimulatory blockade.

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