A method for assessing risk of losing a transplanted organ by a patient having an episode of acute rejection of the transplanted organ is described. The method includes obtaining from the patient a cell sample from the transplanted organ or peripheral blood, determining a level of FOXP3 in the cell sample, and correlating the level with the risk of loss of the transplanted organ, wherein, compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a significantly lower level of FOXP3 in the cell sample from the peripheral blood correlates with a decreased risk of loss of the transplanted organ.

\[ r_s = -0.42, \ P = 0.01 \]

Days from Transplantation to Biopsy (log scale)
METHODS OF USING FOXP3 LEVELS TO PREDICT THE OUTCOME OF ORGANS UNDERGOING ACUTE REJECTION

[0001] This application asserts priority to U.S. Provisional Application Ser. No. 60/848,040 filed on Sep. 26, 2006, the specification of which is hereby incorporated by reference in its entirety.

[0002] The invention described in this application was made with funds from the National Institutes of Health, Grant Numbers R01 A151652 and A160706. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Acute rejection of an organ, transplanted from one human to another, is an important risk factor for allograft failure. The outcome of acute rejection is, however, difficult to predict.

[0004] Currently, observation of histologic features in allograft tissue obtained by core needle biopsy is the best predictor whether an acute rejection will respond to anti-rejection therapy. However, the invasive procedure of allograft biopsy is associated with complications such as bleeding, arteriovenous fistula, and even graft loss. Thus, there is a need for a non-invasive method for determining whether a patient suffering from acute rejection of a transplant organ is at risk of loss of the transplanted organ.

SUMMARY OF THE INVENTION

[0005] The above need has been met by the present invention, which provides in one embodiment a method for assessing risk of losing a transplanted organ by a patient having an episode of acute rejection of the transplanted organ, the method comprising obtaining from the patient a cell sample from the transplanted organ or from peripheral blood, determining a level of FOXP3 in the cell sample, and correlating the level with the risk of loss of the transplanted organ, wherein, compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a significantly lower level of FOXP3 in the cell sample from peripheral blood correlates with a decreased risk of loss of the transplanted organ, or a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with an increased risk of loss of the transplanted organ.

[0006] In another embodiment of the present invention, a method for assessing likelihood of responding to anti-rejection treatment by a patient having an episode of acute rejection of the transplanted organ, the method comprises obtaining from the patient a cell sample from the transplanted organ or from peripheral blood, determining a level of FOXP3 in the cell sample; and correlating the level with the likelihood of responding to anti-rejection treatment wherein, compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a significantly lower level of FOXP3 in the cell sample from peripheral blood correlates with an increased likelihood of responding to anti-rejection treatment, or a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with a decreased likelihood of responding to anti-rejection treatment.

[0007] In another embodiment of the present invention, a method for assessing the likelihood of reversing an acute rejection of a transplanted organ by a patient having an episode of acute rejection of the transplanted organ, the method comprises obtaining from the patient a cell sample from the transplanted organ or from peripheral blood, determining a level of FOXP3 in the cell sample, correlating the level with the likelihood of reversing acute rejection of the transplanted organ, wherein, compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a significantly lower level of FOXP3 in the cell sample from peripheral blood correlates with an increased likelihood of reversing acute rejection of the transplanted organ, or a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with a decreased likelihood of reversing acute rejection of the transplanted organ.

BRIEF DESCRIPTION OF THE FIGURES

[0008] FIG. 1. Level of FOXP3 mRNA in urinary cells. Box plots show the 10th, 25th, 50th (median), 75th, and 90th percentile values for log-transformed ratios of mRNA copies to 18S rRNA copies for FOXP3, CD25, CD3ε, and perforin in urine samples obtained from 36 subjects with biopsy-confirmed acute rejection, 18 subjects with biopsy-confirmed chronic allograft nephropathy, and 29 subjects with stable graft function and normal biopsy results. The levels of mRNA for FOXP3, CD25, CD3ε, and perforin were higher in the urinary cells from subjects with acute rejection than in the subjects with chronic allograft nephropathy or normal biopsy results. P values are based on the Kruskal-Wallis test with the log-transformed mRNA levels treated as the dependent variable. Dunn’s multiple-comparison test showed that levels of FOXP3 mRNA in the acute-rejection group were higher than those in both the group with chronic allograft nephropathy (P<0.05) and the group with normal biopsy results (P<0.01) (Panel A). CD25 mRNA levels were higher in the acute-rejection group than in both the group with chronic allograft nephropathy (P<0.001) and the group with normal biopsy results (P<0.001) (Panel B). CD3ε mRNA levels were higher in the acute-rejection group than in both the group with chronic allograft nephropathy (P<0.01) and the group with normal biopsy results (P<0.05) (Panel C). Perforin mRNA levels were higher in the acute-rejection group than in both the group with chronic allograft nephropathy (P<0.001) and the group with normal biopsy results (P<0.001) (Panel D). In all cases, log-transformed level, normalized for 18S rRNA, are shown.

[0009] FIG. 2. Correlation between Levels of FOXP3 mRNA in Urinary Cells and Reversal of an Episode of Acute Rejection. Box plots show the 10th, 25th, 50th (median), 75th, and 90th percentiles for levels of mRNA for FOXP3, CD25, CD3ε, and perforin in urine samples obtained from 26 subjects with successful reversal of acute rejection (classified as reversible and defined by the return of serum creatinine levels to within 15 percent of prererejection levels within four weeks after the initiation of antirejection treatment) and 10 patients without reversal of acute rejection (nonreversible).
The levels of mRNA for FOXP3 but not for CD25, CD3e, and perforin were significantly higher in subjects with reversible acute rejection than in subjects with nonreversible acute rejection. Two-tailed P values are based on the Mann-Whitney test. In all cases, log-transformed levels, normalized for 18S rRNA, are shown.

Figs. 3, 4, and 5. Receiver-Operating-Characteristic (ROC) Curves for mRNA Levels. The fraction of true positive results (sensitivity) and false positive results (1-specificity) for levels of mRNA for FOXP3, CD25, CD3e, and perforin, each normalized for 18S rRNA, as predictors of failure of the transplanted organ are shown. The ROC curves were constructed for mRNA levels in a control patient group compared to a control patient group with acute rejection (Panel A), compared to a group with acute rejection (Panel B), and compared to a control group with acute rejection (Panel C). The ROC curves show that mRNA levels for FOXP3, CD25, CD3e, and perforin are significantly higher in the cell sample from the peripheral blood in the group with acute rejection than in the control group. A P value of 0.5 is no better than that expected by chance (null hypothesis), and a P value of 0.1 reflects a perfect indicator. Of the four mRNA measures, only FOXP3 predicts successful reversal significantly better than chance (P = 0.001).

In one aspect, the invention provides a method for assessing risk of losing a transplanted organ by a patient suffering from acute rejection of the transplanted organ. In another aspect, the invention provides a method for assessing the likelihood of reversing an acute rejection of a transplanted organ by a patient suffering from acute rejection of the organ.

Each of the methods of the present invention can be used alone, or in combination with one or more, or all, of the other methods.

The first step in the methods of the present invention comprises obtaining from the patient a cell sample from the transplanted organ or from peripheral blood. Suitable methods for obtaining a cell sample are provided below in the “General Methods” section.

The second step in the methods comprises determining the level of FOXP3 in the cell sample. The determination of the level of FOXP3 in the cell sample can be made by any method known to those skilled in the art. In one embodiment, the level of FOXP3 mRNA or the corresponding level of cDNA is determined. In another embodiment, the level of FOXP3 protein is determined. Suitable methods are provided below in the “General Methods” section.

Method for Accessing Risk of Loss of Transplanted Organ

In this aspect of the present invention, the first and second steps of the method are described above. The next step in the method comprises correlating the level of FOXP3 with the risk of loss of the transplanted organ.

When compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a level of FOXP3 during an episode of acute rejection that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 during an episode of acute rejection that is not significantly lower in the cell sample from peripheral blood correlates with an increased risk of loss of the transplanted organ.

The patient is any human having an episode of acute rejection of a transplanted organ. Any organ suitable for transplantation can be susceptible to an episode of acute rejection. Examples of such organs include kidney, heart, liver, lung, intestines, pancreas, pancreatic islets, etc.

An episode of acute rejection of an organ can be caused by an antibody-mediated or cell-mediated immune response. The cells involved in a cell-mediated immune response include, for example, activated cytotoxic T cells. An episode of acute rejection typically occurs within fourteen days, more typically within ten days, and even more typically within five days after a transplant if the patient is not taking an immunosuppressant drug.

However, most if not all transplant patients are given immunosuppressant drugs. Thus, an episode of acute rejection generally occurs within about one year of a transplanted organ, more generally within about nine months, even more generally within about six months, and most generally within about three months after transplant of an organ. Acute rejection, however, can occur at any time during the life of a transplanted organ. Further, a patient can have more than one episode of acute rejection of a transplanted organ.

In one aspect, the invention provides a method for assessing risk of losing a transplanted organ by a patient suffering from acute rejection of the transplanted organ. In another aspect, the invention provides a method for assessing the likelihood of reversing an acute rejection of a transplanted organ by a patient suffering from acute rejection of the organ.

Each of the methods of the present invention can be used alone, or in combination with one or more, or all, of the other methods.

The first step in the methods of the present invention comprises obtaining from the patient a cell sample from the transplanted organ or from peripheral blood. Suitable methods for obtaining a cell sample are provided below in the “General Methods” section.

The second step in the methods comprises determining the level of FOXP3 in the cell sample. The determination of the level of FOXP3 in the cell sample can be made by any method known to those skilled in the art. In one embodiment, the level of FOXP3 mRNA or the corresponding level of cDNA is determined. In another embodiment, the level of FOXP3 protein is determined. Suitable methods are provided below in the “General Methods” section.

Method for Accessing Risk of Loss of Transplanted Organ

In this aspect of the present invention, the first and second steps of the method are described above. The next step in the method comprises correlating the level of FOXP3 with the risk of loss of the transplanted organ.

When compared to a control level, a significantly greater level of FOXP3 in the cell sample from the trans-
planted organ or a significantly lower level of FOXP3 in the cell sample from the peripheral blood correlates with a decreased risk of loss of the transplanted organ. The decreased risk varies in different patients, and the type of organ transplanted. Generally, the decreased risk is at least about 25%, at least about 50%, at least about 75%, or at least about 90%.

[0025] When compared to a control level, a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with an increased risk of loss of the transplanted organ. The increased risk varies in different patients, and the organ transplanted. Generally, the increased risk is at least about 25%, at least about 50%, at least about 75%, or at least about 90%.

[0026] When the transplanted organ is a kidney, the method for assessing risk of a patient suffering from acute rejection optionally further comprises determining the patient's serum creatinine protein level. The determination of the level of serum creatinine can be made by any method known to those skilled in the art. Suitable methods are provided below in the "General Methods" section below.

[0027] The next step in this embodiment comprises correlating the level of serum creatinine in peripheral blood with risk of loss of the transplanted organ. A significantly greater level of serum creatinine in peripheral blood correlates with an increased risk of loss of the transplanted kidney. A level of serum creatinine in peripheral blood that is not significantly greater correlates with a decreased risk of loss of the transplanted kidney.

[0028] Generally, the level of serum creatinine in peripheral blood is considered to be significantly greater if the level is at least about 25% greater than the level of creatinine in a control sample.

[0029] In this embodiment, a control sample is typically the level of serum creatinine in peripheral blood of a healthy person or a person with a well-functioning (e.g., stable) transplant. For example, the normal level of serum creatinine in a healthy person or a person with a well-functioning transplant is generally about 0.8-1.6 milligrams/deciliter. In either case, the person may be the patient or a person different from the patient.

[0030] It is not necessary to determine the level of creatinine in a control sample every time the method is conducted. For example, the serum creatinine level from the patient can be compared to that of one or more previously determined control samples or to a level recognized by the physician or clinician conducting the method, or by a consensus of medical and/or clinical practitioners.

[0031] In another embodiment, the method further comprises informing the patient whether the patient is at decreased or increased risk of loss of the transplanted organ. The information that a patient is at risk of loss of a transplanted organ is useful. Such patients can be prescribed and/or administered a treatment to prevent loss of the transplanted organ.

[0032] In one embodiment, the treatment comprises administering to the patient an effective amount of a pharmaceutical composition to prevent loss of the transplanted organ. Such pharmaceutical compositions are well known to those skilled in the art, and include, for example a steroid pulse, an antibody, etc.

[0033] For example, a steroid pulse therapy can include the administration for three to six days of a high dose corticosteroid (e.g., greater than 100 mg). An example of an antibody therapy includes the administration for seven to fourteen days of the polyclonal antibody Thymoglobulin or the monoclonal antibody, OKT3.

[0034] Another example of a treatment that can be administered is plasmapheresis. Plasmapheresis is a process in which the fluid part of the blood (i.e., plasma) is removed from blood cells. Typically, the plasma is removed by a device known as a cell separator. The cells are generally returned to the person undergoing treatment, while the plasma, which contains antibodies, is discarded.

Method for Assessing Likelihood of Responding to Anti-Rejection Treatment

[0035] In this aspect of the present invention, the first and second steps of the method are described above. The next step in the method comprises correlating the level of FOXP3 with the likelihood of the patient in responding to anti-rejection treatment.

[0036] When compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a significantly lower level of FOXP3 in the cell sample from peripheral blood correlates with an increased likelihood of responding to anti-rejection treatment. The increased likelihood varies in different patients and the type of organ transplanted. Generally, the increased likelihood of responding to anti-rejection treatment means the likelihood is increased by at least 25%, more preferably by at least 50%, and even more preferably by at least 75%. Optionally, the patient responds to anti-rejection therapy such that any risk of complete failure of the transplanted organ is completely eliminated.

[0037] When compared to a control level, a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with a decreased likelihood of responding to anti-rejection treatment. The decreased likelihood varies in different patients, and the type of organ transplanted. Generally, the decreased likelihood of responding to anti-rejection treatment means the likelihood is decreased by at least 25%, by at least 50%, and even by at least 75%.

[0038] Examples of anti-rejection treatments include those treatment described above for preventing rejection of a transplanted organ.

Method for Assessing Likelihood of Reversing Acute Rejection

[0039] In this aspect of the present invention, the first and second steps of the method are described above. The next step in the method comprises correlating the level of FOXP3 with the likelihood of reversing acute rejection of the transplanted organ.

[0040] When compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a significantly lower level of FOXP3 in the cell sample from peripheral blood correlates with an increased likelihood of reversing acute rejection of the transplanted organ. The increased likelihood varies in different patients and the type of organ transplanted. Generally, the increased likelihood of reversing acute rejection of the trans-
planted organ means that the likelihood is increased by at least about 25%, more preferably by at least about 50%, and even more preferably by at least about 75%. Optimally, rejection of the transplanted organ is completely eliminated.

[0041] When compared to a control level, a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with a decreased likelihood of reversing acute rejection of the transplanted organ. The decreased likelihood varies among different patients and the type of organ transplanted. Generally, the decreased likelihood of reversing acute rejection means the likelihood is decreased by at least about 25%, by at least about 50%, and even by at least about 75%.

General Methods

[0042] A cell sample from a transplanted organ or peripheral blood can be obtained from a patient by any method known to those in the art. Examples of such cell samples include transplant tissue biopsy, blood, urine, bile, bronchoalveolar lavage fluid, and pericardial fluid. Suitable methods include, for example, venous puncture of a vein to obtain a blood sample and collection of a urine specimen.

[0043] Any method known to those in the art can be employed for determining the level of FOXP3 mRNA. Typically, total RNA, which includes mRNA, is isolated. RNA can be isolated from the sample by any method known to those in the art. For example, commercial kits, such as the TRI Reagent® commercially available from Molecular Research Center, Inc. (Cincinnati, Ohio), can be used to isolate RNA.

[0044] The quantification of FOXP3 mRNA from total mRNA from the biological sample can be performed by any method known to those in the art. For example, kinetic, quantitative PCR involves reverse transcribing FOXP3 mRNA by using reverse-transcriptase polymerase chain reaction (RT-PCR) to obtain FOXP3 cDNA. The cDNA can then, for example, be amplified by PCR followed by quantitation using a suitable detection apparatus. See example 1 below for a description of the quantitation of FOXP3 mRNA by kinetic, quantitative PCR.

[0045] Generally, the isolated FOXP3 mRNA may be amplified by methods known in the art. Amplification systems utilizing, for example, PCR or RT-PCR methodologies are known to those skilled in the art. For a general overview of amplification technology, see, for example, Dieffenbach et al., *PCR Primer: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (1995). For example, levels of FOXP3 mRNA can be determined using kinetic, quantitative PCR.

[0046] An alternative method for determining the level of FOXP3 mRNA includes the use of molecular beacons and other labeled probes useful in, for example multiplex PCR. In a multiplex PCR assay, the PCR mixture contains primers and probes directed to the FOXP3 PCR product. Typically, a single fluorochrome is used in the assay. The molecular beacon or probe is detected to determine the level of FOXP3 mRNA. Molecular beacons are described, for example, by Tyagi and Kramer (Nature Biotechnology 14, 303-308, 1996) and by Andrus and Nichols in U.S. Patent Application Publication No. 20040053284.

[0047] Another method includes, for instance, quantifying cDNA (obtained by reverse transcribing the FOXP3 mRNA) using a fluorescence-based real-time detection method, such as the ABI PRISM 7700 or 7900 Sequence Detection System [TaqMan®] commercially available from Applied Biosystems, Foster City, Calif. or similar system as described by Heid et al., (Genome Res. 1996; 6:986-994) and Gibson et al. (Genome Res. 1996; 6:995-1001).

[0048] Any method known in the art can be used for determining the protein level of FOXP3 in the cell sample from a transplanted organ or peripheral blood, or serum creatinine levels from peripheral blood.

[0049] Suitable methods for determining protein levels include an ELISA and a standard blot. Briefly, these assays are normally based on incubating an antibody specific to the protein with a sample suspected of containing the protein, and detecting the presence of a complex between the antibody and the protein.

[0050] Alternatively, commercial kits can be utilized. An example of a commercial kit for determining creatinine level is the QuantiChrom™ Creatinine Assay Kit from BioAssay Systems (Hayward, Calif.).

[0051] Generally, the level of FOXP3 in a cell sample is significantly greater if the gene expression of FOXP3 is heightened. For example, a discriminatory level for heightened gene expression (e.g., the baseline magnitude of gene expression) of FOXP3 is defined as the mean±95% confidence interval of a group of values observed in nonrejecting transplants (e.g., control values, i.e., control levels). The group of values as used herein includes, for example, a minimum of at least about 2 values, more preferably a minimum of at least about 10 values, most preferably a minimum of at least about 20 values. The group of values as used herein includes, for example, a maximum of at most about 500 values, more preferably a maximum of at most about 100 values, most preferably a maximum of at most about 50 values.

[0052] Heightened gene expression of FOXP3 is considered to be significantly greater if the value is greater than the mean±95% confidence interval of a group of values observed in nonrejecting transplants. Similarly, the level of FOXP3 in the cell sample is considered to be significantly lower if the FOXP3 value is lower than the mean±95% confidence interval of a group of values observed in nonrejecting transplants.

[0053] The level of FOXP3 is typically considered not significantly greater if the level of FOXP3 in a cell sample is not greater than the mean±95% confidence interval of a group of values observed in nonrejecting transplants. The level of FOXP3 is normally considered not significantly lower if the level in a cell sample is not lower than the mean±95% confidence interval of a group of values observed in nonrejecting transplants.

[0054] Statistical analysis in the above mean±95% confidence interval of a group of values observed in nonrejecting transplants was performed with a x² test.

[0055] In another embodiment, the level of FOXP3 in a cell sample is significantly greater if the log-transformed mean (±SE) ratio of FOXP3 mRNA copies to 18S-rRNA copies is higher relative to a control ratio in nonrejecting organs, as determined by the Kruskal-Wallis test. For example, a significantly greater ratio is typically at least about ±SE 3.0, more typically between ±SE 3.0 and 5.0, and most typically between ±SE 3.8 and 4.7.

[0056] Similarly, the level of FOXP3 in a cell sample is significantly lower if the log-transformed mean (±SE) ratio of FOXP3 mRNA copies to 18S-rRNA copies is reduced relative to a control ratio (i.e., control values, control levels) in nonrejecting organs, as determined by the Kruskal-Wallis
test. For example, a typical nonrejection organ control ratio is not more than about 2.5, more typically 1.0 to 2.5, and most typically from 1.3 and 2.0.

[0057] In yet another embodiment, the control values (control levels) observed in nonrejecting organs, e.g., kidneys, may be defined as the level of FOXP3 of the same patient before the organ transplant; the average level of FOXP3 in patients of similar age, gender, race, graft-donor source, Banff histologic grade, or initial antirejection treatment as the patient; or a value for the level of FOXP3 accepted in the art.

[0058] In an embodiment of the invention, generally, the level of FOXP3 when compared to a control level may be increased by at least about 10%, at least about 50%, or at least about 100%. The level of FOXP3 when compared to a control level may be decreased by at least about 10%, at least about 50%, or at least about 100% lower than the level of FOXP3 in a control sample.

[0059] A control sample is typically the level of FOXP3 from a healthy person or a person with a well-functioning (e.g., stable) transplanted organ. A well-functioning (e.g., stable) transplanted organ may be defined as a transplanted organ without acute rejection, and preferably a transplanted organ that has not developed transplant dysfunction or morphologic evidence of transplant injury in areas of the transplant. For example, a stable functioning kidney transplant may be defined as having a serum creatinine concentration that has not changed by more than approximately 0.2 mg per deciliter during the seven days before and the seven days after collection of the biologic specimen for FOXP3 measurements.

[0060] It is not necessary to determine the level of FOXP3 mRNA or FOXP3 protein in a control sample every time the method is conducted. For example, the FOXP3 levels in the cell sample from the transplanted organ or in the cell sample from the peripheral blood can be compared to that of one or more previously determined control samples or to a level recognized by the physician or clinician conducting the method of a consensus of medical and/or clinical practitioners.

EXAMPLES
Example 1

Methods

[0061] Study Cohorhis. Urine samples from 83 kidney-transplant recipients were examined. In this group were 36 subjects with graft dysfunction (mean ±SD) creatinine level, 3.6±2.4 mg per deciliter [318.2±212.2 pmol per liter] and biopsy-confirmed acute rejection (mean age: 41±12 years; 15 men and 21 women; 13 white, 12 black, and 11 with other racial or ethnic backgrounds; with 20 living and 16 deceased donors), 29 subjects with stable allograft function (mean creatinine level, 1.4±0.4 mg per deciliter [123.8±35.4 μmol per liter]) and normal allograft biopsy (mean age, 44±14 years; 15 men and 14 women; 12 white, 4 black, and 13 with other racial or ethnic backgrounds; with 26 living and 3 deceased donors), and 18 subjects with allograft dysfunction (mean creatinine level, 3.1±1.6 mg per deciliter [274.0±141.4 μmol per liter]) and biopsies classified as indicating chronic allograft nephropathy (mean age, 52±12 years; 9 men and 9 women; 9 white, 2 black, and 7 with other racial or ethnic backgrounds; with 5 living and 13 deceased donors).

[0062] Seventy-five of the 83 urine specimens were collected before the biopsy procedure, and 8 samples were obtained after the procedure. Formalin-fixed, paraffin-embedded renal-biopsy specimens were stained with hematoxylin and eosin, periodic acid-Schiff, and Masson’s trichrome stains and were scored with the use of the Banff ’97 classification by a pathologist who was blinded to the results of molecular studies. Immunosuppression consisted of a calcineurin inhibitor-based regimen (cyclosporine or tacrolimus), with the administration of glucocorticoids, antilymphocyte antibodies (muromonab-CD3 [OKT3] or antilymphocyte globulin), or both for the treatment of acute rejection.

[0063] Quantitation of mRNA by Kinetic Quantitative PCR. Total RNA was isolated from urine-cell pellets, quantified, and reverse transcribed to complementary DNA (cDNA). Oligonucleotide primers and fluorogenic probes were designed and synthesized for the measurement of mRNA levels of FOXP3, CD25, CD3e, perforin, and 18S ribosomal RNA (rRNA).

[0064] PCR analysis was performed by a two-step process, a preamplification step followed by measurement of mRNA with an ABI Prism 7700 system. Transcript levels were calculated by a standard curve method, and mRNA copy numbers were normalized with the use of 18S rRNA copy numbers (the number of mRNA copies in 1 pg of RNA divided by the number of 18S rRNA copies in 1 fg of RNA). When no detectable level of a transcript was found, a value equal to half the minimum observed 18S-normalized level was assigned. For an estimation of group means, this method is considered a reasonable substitute for the value of zero or the minimum detected value; moreover, the nonparametric statistical tests of group differences reported below are not affected by the choice of value.

[0065] Statistical Analysis. The levels of mRNA for FOXP3, CD25, CD3e, perforin, and 18S rRNA deviated from a normal distribution (P<0.001), but a log transformation substantially reduced the positive skew. We used the 18S-normalized level as the dependent variable in a Kruskal-Wallis test to identify any differences among the group with acute rejection, the group with chronic allograft nephropathy, and the group with normal biopsy results and then used Dunn’s test for multiple comparisons. The Mann-Whitney test, equivalent to the Kruskal-Wallis test when applied to two groups, was used when mRNA levels were compared between two groups. Spearman’s rank-order correlations were used to test for a monotonic association of the 18S-adjusted mRNA transcript levels with serum creatinine levels and time (in days) from kidney transplantation to biopsy. An episode of acute rejection was classified as reversible if the serum creatinine level returned to within 15 percent of the prererejection level within four weeks after the initiation of antirejection treatment. A second end point was the loss of the graft during the first six months after the diagnosis of acute rejection. We used receiver-operating-characteristic (ROC) curves to analyze mRNA levels in order to determine the cutoff points that yielded the highest combined sensitivity and specificity for predicting the outcome of an episode of acute rejection.

Example 2

Levels of FOXP3 mRNA in Urinary Cells

[0066] The log-transformed mean ±SE ratio of FOXP3 mRNA copies to 18S-rRNA copies in urinary cells was 3.8±0.5 in the 36 subjects with acute rejection and was higher
than the levels in both the 18 subjects with chronic allograft nephropathy (1.3±0.7) and the 29 subjects with normal biopsy results (1.6±0.4, P<0.001 by the Kruskal-Wallis test) (FIG. 1A). Among the three groups, the 18S-normalized, log-transformed mRNA levels of CD25 (6.9±0.4, 4.0±0.5, and 2.8±0.6, respectively; P<0.001), CD3e (8.2±0.4, 4.3±0.5, and 1.6±0.5; P<0.001), and perforin (7.6±0.4, 4.5±0.4, and 2.8±0.4; P<0.001) were also highest in the acute-rejection cohort (FIGS. 1B, 1C, and 1D).

Example 3

FOX3 mRNA Levels and Disease Severity

We observed a significant inverse relationship between the levels of FOX3 mRNA and serum creatinine measured during an episode of acute rejection (Spearman’s correlation coefficient r_s=−0.38, P=0.02). By contrast, serum creatinine levels were not significantly related to mRNA levels of CD25 (r_s=−0.01, P=0.93), CD3e (r_s=−0.11, P=0.54), or perforin (r_s=−0.23, P=0.18) in the acute-rejection group. Also, the mean (±SE) serum creatinine level in the 16 subjects with acute rejection of Banff grade IA (moderate tubulitis) did not differ significantly from that of the 20 subjects with grade IB (severe tubulitis) or more (3.3±0.6 mg per deciliter [291.7±53.0 μmol per liter] as compared with 3.8±0.6 mg per deciliter [318.2±53.0 μmol per liter], P=0.57).

There was no correlation between the levels of FOX3 mRNA and serum creatinine that were measured in the group with chronic allograft nephropathy (r_s=−0.02, P=0.93) or the group with normal biopsy results (r_s=−0.08, P=0.67).

Example 4

FOX3 mRNA Levels and Reversal of Acute Rejection

Twenty-six of the 36 episodes of acute rejection qualified as successfully reversed; the remaining 10 did not. Levels of FOX3 mRNA in urinary cells were significantly higher in the group with successful reversal than in the group without reversal (mean ±SE level 4.7±0.5 and 1.5±0.7, respectively; P<0.001) (FIG. 2A). In the two groups, the levels of mRNA for CD25 (7.3±0.4 and 6.0±0.9, P=0.22), CD3e (8.5±0.5 and 7.4±0.8, P=0.35), and perforin (7.8±0.5 and 7.3±0.7, P=0.43) were not informative of outcome (FIGS. 2B, 2C, and 2D).

The ROC curves (FIG. 3) show the fraction of true positive results (sensitivity) and false positive results (1-specificity) for various cutoff levels of mRNA for FOX3, CD25, CD3e, and perforin. The log-transformed threshold that gave the maximal sensitivity and specificity for FOX3 mRNA was 3.46; using the cutoff value of 3.46 derived from the data, the FOX3 mRNA level predicted rejection reversal with a sensitivity of 90 percent and a specificity of 73 percent (P=0.001) (FIG. 3A). The levels of mRNA for CD25, CD3e, and perforin were not predictive of reversal of acute rejection (FIGS. 3B, 3C, and 3D).

Successful reversal of acute rejection, as compared with unsuccessful reversal, was not predicted by the subjects’ age (mean ±SD, 41±2.2 years and 42±2.4 years, respectively; P=0.52), sex (4 men and 6 women vs. 11 men and 15 women, P=0.90), race (3 white, 6 black, and 1 with other race or ethnic background vs. 10 white, 6 black, and 10 with other race or ethnic background; P=0.08), graft-donor source (15 living and 11 deceased vs. 5 living and 5 deceased, P=0.68), Banff histologic grade (11 with IA and 15 with >IA vs. 5 with IA and 5 with >IA, P=0.68), or initial antirejection treatment (24 with glucocorticoids and 2 with antilymphocyte antibodies vs. 7 with glucocorticoids and 3 with antilymphocyte antibodies, P=0.12). Among subjects with successful reversal, as compared with those with unsuccessful reversal, serum creatinine levels (median levels, 2.3 mg per deciliter and 6.5 mg per deciliter, respectively; P<0.001) and the time from kidney transplantation to the development of acute rejection (median time, 82 days and 523 days, respectively; P=0.008) were lower. In logistic-regression analyses predicting nonresponse, levels of FOX3 mRNA in urinary cells remained significant after statistical control for serum creatinine level (P=0.04) and the time from transplantation to rejection (P=0.02).

A linear combination of levels of FOX3 mRNA and creatinine was a better predictor of rejection reversal (90 percent sensitivity and 96 percent specificity) than FOX3 mRNA levels alone (90 percent sensitivity and 73 percent specificity) or serum creatinine levels alone (85 percent sensitivity and 90 percent specificity).

Example 5

FOX3 mRNA Levels and Allograft Failure

Ten of the 36 subjects with acute rejection lost their grafts within six months after the incident episode of acute rejection, and 9 of those 10 subjects did not respond to the initial antirejection therapy. Renal-allograft recipients with a failed allograft within six months after the episode of acute rejection had significantly lower FOX3 mRNA levels in their urinary cells than the 26 subjects who had a functioning allograft (2.0±0.8 and 4.5±0.5, respectively; P=0.001). In the two groups, the levels of mRNA for CD25 (6.6±0.7 and 7.1±0.5, P=0.33), CD3e (7.9±0.7 and 8.3±0.5, P=0.76), and perforin (7.8±0.6 and 7.6±0.5, P=0.90) did not predict allograft loss.

The rate and relative risk of graft failure within six months after an episode of acute rejection, for thirds of each mRNA measure, are shown in FIG. 4. At the highest third of FOX3 mRNA levels, the graft failure rate was 8 percent; at the middle third, the graft failure rate was 25 percent and the relative risk was 3; and at the lowest third, the graft failure rate was 50 percent and the relative risk was 6 (P<0.02 by the chi-square test for linear trend) (FIG. 4A). In contrast, the rate of graft failure after an episode of acute rejection did not differ significantly across the thirds of mRNA levels for CD25, CD3e, and perforin (FIGS. 4B, 4C, and 4D).

Graft failure as compared with graft success was not predicted by the subjects’ age (mean ±SD, 39±4.2 years and 42±2.4 years, respectively; P=0.52), sex (4 men and 6 women vs. 11 men and 15 women, P=0.90), race (3 white, 6 black, and 1 with other race or ethnic background vs. 10 white, 6 black, and 10 with other race or ethnic background; P=0.08), graft-donor source (5 living and 5 deceased vs. 15 living and 11 deceased, P=0.68), Banff histologic grade (5 with IA and 5 with >IA vs. 11 with IA and 15 with >IA, P=0.68), or initial antirejection treatment (24 with glucocorticoids and 2 with antilymphocyte antibodies vs. 7 with glucocorticoids and 3 with antilymphocyte antibodies, P=0.12). In subjects with graft failure, as compared with subjects with graft success, serum creatinine levels (median levels, 6.5 mg per deciliter [574.6 μmol per liter] and 2.3 mg per deciliter
[203.3 μmol per liter, respectively; P<0.001) and the time from kidney transplantation to the development of acute rejection (median time, 562 days and 82 days; P=0.003) were significantly greater. In a logistic-regression analysis, FOXP3 mRNA levels became nonsignificant after control for serum creatinine levels (P=0.13) or time between transplantation and rejection (P=0.09).

[0076] A linear combination of levels of FOXP3 mRNA and creatinine was a better predictor of graft failure (90 percent sensitivity and 92 percent specificity) than were either FOXP3 mRNA levels alone (80 percent sensitivity and 69 percent specificity) or serum creatinine levels alone (85 percent sensitivity and 90 percent specificity).

Example 6

FOXP3 mRNA Levels and Time to Acute Rejection

[0077] Late acute rejection (acute rejection occurring at least three months after transplantation) results in an outcome that is inferior to that of early acute rejection. We found a strong inverse relationship between levels of FOXP3 mRNA in urinary cells and the time from kidney transplantation to the development of acute rejection (r=−0.42, P=0.01) (FIG. 5A). Levels of FOXP3 mRNA in urinary cells were lower in 11 urine specimens from patients with late acute rejection than in 25 specimens from patients with early acute rejection (mean ±[SE] level, 2.5±0.6 and 4.7±0.5; P=0.009). CD25 mRNA levels also showed an inverse relation (r=−0.45, P=0.006) (FIG. 5B), and the levels were lower during late rejection than during early acute rejection (5.8±0.8 and 7.4±0.4, P=0.07). There was no inverse relation between the time from kidney transplantation to the development of acute rejection and the mRNA levels of CD3ε (r=−0.26, P=0.12) (FIG. 5C) or perforin (r=−0.02, P=0.91) (FIG. 5D). There was also no correlation between the serum creatinine levels at the time of acute rejection and the time from kidney transplantation to the development of acute rejection (r=−0.23, P=0.17).

Example 7

FOXP3 Levels in Cell Samples from Peripheral Blood are Predictive of Acute Rejection Outcome

[0078] To determine whether peripheral blood cell FOXP3 mRNA levels are informative of human renal allograft status, peripheral blood, using PAXgene RNA tubes, were collected from 38 renal allograft recipients; 11 subjects with acute rejection and 27 subjects with normal protocol biopsies and stable graft function. Peripheral blood cell mRNA for FOXP3 and mRNA for a constitutively expressed gene 18S ribosomal RNA (18S rRNA) were measured using kinetic quantitative PCR assay. The level of expression, normalized using 18S rRNA copy numbers and log transformed to reduce the positive skew, was correlated with renal allograft status.

[0079] Data analyses demonstrated that peripheral blood cell FOXP3 mRNA levels are significantly lower during an episode of acute rejection as compared to levels observed in samples from subjects with normal biopsies and stable graft function (P=0.04, Mann Whitney test). Furthermore, graft outcome following an episode of acute rejection was predicted by peripheral blood cell FOXP3 mRNA levels; there was no graft loss in the lowest tertile, 3 of 4 grafts were lost in the middle tertile, and 2 of 5 grafts failed in the highest tertile.

What is claimed is:

1. A method for assessing risk of losing a transplanted organ by a patient having an episode of acute rejection of the transplanted organ, the method comprising:
   (a) obtaining from the patient a cell sample from the transplanted organ or from peripheral blood;
   (b) determining a level of FOXP3 in the cell sample; and
   (c) correlating the level with the risk of loss of the transplanted organ,
   wherein, compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a significantly lower level of FOXP3 in the cell sample from the peripheral blood correlates with a decreased risk of loss of the transplanted organ, or a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with an increased risk of loss of the transplanted organ.

2. A method according to claim 1, wherein the level of FOXP3 is determined by measuring the level of FOXP3 in RNA.

3. A method according to claim 1, wherein the level of FOXP3 is determined by measuring the level of FOXP3 protein.

4. A method according to claim 1, wherein the method further comprises assessing the likelihood of the patient's responding to anti-rejection treatment, wherein, compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ, or a significantly lower level of FOXP3 in the cell sample from the peripheral blood correlates with an increased likelihood of responding to anti-rejection treatment, and a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with a decreased likelihood of responding to anti-rejection treatment.

5. A method according to claim 5, wherein the transplanted organ is a kidney.

6. A method according to claim 3, wherein the cell sample from the kidney is urine.

7. A method according to claim 1, wherein the transplanted organ is a heart.

8. A method according to claim 1, wherein the transplanted organ is a liver.

9. A method according to claim 1, wherein the transplanted organ is a lung.

10. A method according to claim 1, wherein the transplanted organ is a pancreas.

11. A method according to claim 1, wherein the transplanted organ is a pancreatic islet.

12. A method according to claim 1, wherein the transplanted organ is intestine.

13. A method according to claim 1, further comprising informing the patient whether the patient is at decreased or increased risk of loss of the transplanted organ.

14. A method according to claim 1, further comprising prescribing to the patient at increased risk of loss of the transplanted organ a treatment to prevent loss of the transplanted organ.

15. A method according to claim 14, wherein the treatment comprises an effective amount of a pharmaceutical composition to prevent loss of the transplanted organ.
16. A method according to claim 12, wherein the pharmaceutical composition comprises a steroid pulse.

17. A method according to claim 13, wherein the steroid is a corticosteroid.

18. A method according to claim 12, wherein the pharmaceutical composition comprises an antibody.

19. A method according to claim 14, wherein the antibody is thymoglobin or OKT3.

20. A method according to claim 14, wherein the treatment comprises plasmapheresis.

21. A method according to claim 5, further comprising determining the patient’s serum creatinine level in peripheral blood, and correlating the level with the risk of loss of the transplanted kidney, wherein, compared to a control level, a significantly greater level of serum creatinine in peripheral blood correlates with an increased risk of loss of the transplanted kidney, or a level of serum creatinine in peripheral blood that is not significantly greater correlates with a decreased risk of loss of the transplanted kidney.

22. A method for assessing likelihood of responding to anti-rejection treatment by a patient having an episode of acute rejection of the transplanted organ, the method comprising:

(a) obtaining from the patient a cell sample from the transplanted organ or from peripheral blood;

(b) determining a level of FOXP3 in the cell sample; and

(c) correlating the level with the likelihood of responding to anti-rejection treatment;

wherein, compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a significantly lower level of FOXP3 in the cell sample from the peripheral blood correlates with an increased likelihood of responding to anti-rejection treatment, or a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with a decreased likelihood of responding to anti-rejection treatment.

23. A method for assessing the likelihood of reversing an acute rejection of a transplanted organ by a patient having an episode of acute rejection of the transplanted organ, the method comprising:

(a) obtaining from the patient a cell sample from the transplanted organ or from peripheral blood;

(b) determining a level of FOXP3 in the cell sample;

(c) correlating the level with the likelihood of reversing acute rejection of the transplanted organ;

wherein, compared to a control level, a significantly greater level of FOXP3 in the cell sample from the transplanted organ or a significantly lower level of FOXP3 in the cell sample from the peripheral blood correlates with an increased likelihood of reversing acute rejection of the transplanted organ, or a level of FOXP3 that is not significantly greater in the cell sample from the transplanted organ or a level of FOXP3 that is not significantly lower in the cell sample from peripheral blood correlates with a decreased likelihood of reversing acute rejection of the transplanted organ.

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