METHOD FOR TREATING OR PREVENTING ISCHEMIA-REPERFUSION INJURY

BACKGROUND OF THE INVENTION

Ischemia-reperfusion injury frequently occurs when the flow of blood to a region of the body is temporarily halted (ischemia) and then re-established (reperfusion). Ischemia-reperfusion injury can occur during certain surgical procedures, such as repair of aortic aneurysms and organ transplantation. Clinically ischemia-reperfusion injury may be manifested by such complications as pulmonary dysfunction, including adult respiratory distress syndrome, renal dysfunction, consumptive coagulopathies including thrombocytopenia, fibrin deposition into the microvasculature and disseminated intravascular coagulopathy, transient and permanent spinal cord injury, cardiac arrhythmias and acute ischemic events, hepatic dysfunction including acute hepatocellular damage and necrosis, gastrointestinal dysfunction including hemorrhage and/or infarction and multisystem organ dysfunction (MSOD) or acute systemic inflammatory response syndromes (SIRS). The injury may occur in the parts of the body to which the blood supply was interrupted, or it can occur in parts fully supplied with blood during the period of ischemia.

International Patent Publication No. WO 96/01318 relates to polypeptides other than interleukin -10 (IL-10) allegedly having one or more properties similar to those of IL-10. Among the very long list of diseases allegedly treatable with these non-IL-10 proteins are tissue damage as a result of "hypoxia/ischemia (infarction: reperfusion)", "ischemia", "reperfusion injury", and "reperfusion syndrome". However, there is no evidence in this publication that the non-IL-10 proteins would actually work for treating all of the diseases in the long list.

SUMMARY OF THE INVENTION

The present invention comprises a method for treating ischemia-reperfusion injury comprising administering to a patient in need of such treatment an effective amount of IL-10. Another aspect of this invention comprises a method for preventing ischemia-reperfusion injury in a patient about to undergo a procedure capable of causing ischemia-reperfusion injury or to a patient who has already undergone such procedure in which ischemia-reperfusion injury has not yet occurred comprising administering to the patient an effective amount of IL-10.

Preferred applications of this invention are preventing ischemia reperfusion injury by administering the IL-10 in conjunction with surgical repair of the thoracic or suprarenal aorta due to aneurysmal disease, but also in conjunction with those surgical procedures that induce or require transient occlusion or bypass of the visceral blood supply via the hepatic, renal and/or enteric arteries secondary to major organ transplant, including liver, kidney, small intestine, and pancreas as well as surgical procedures that result in the transient reduction or prevention of blood flow to the viscera including hepatic and biliary surgical resections, total or partial pancreatectomy (Whipple procedure), total and partial gastrectomy, esophagectomy, colorectal surgery, vascular surgery for mesenteric vascular disease, or abdominal insufflation during laparoscopic surgical procedures.

Additional applications include blunt or penetrating trauma that results in interrruption of blood flow to the visceral organs including those arising from penetrating wounds to the abdomen resulting from gun shot wounds, stab wounds or from penetrating wounds or blunt abdominal trauma secondary to deacceleration injury and/or motor vehicle accidents. Other preferred applications include diseases or procedures that result in systemic hypotension that either disrupts or decreases the flow of blood to the visceral organs, including hemorrhagic shock due to blood loss, cardiogenic shock due to myocardial infarction or cardiac failure, neurogenic shock or anaphylaxis.

Further applications of this invention include preventing or treating lower torso or extremity ischemia reperfusion injury by administering IL-10 in conjunction with surgical procedures that induce or require transient occlusion or bypass of the blood supply to the torso or the upper or lower extremities. This application is particularly relevant to the practice of vascular surgery that encompasses controlled periods of visceral, torso, and extremity ischemia

followed by reperfusion. Procedures which involve such ischemia-reperfusion include but are not limited to repair of abdominal aortic aneurysms, aortic femoral, popliteal or tibial bypass for claudication or limb threatening ischemia, repair of popliteal or femoral aneurysms, bypass, thrombectomy or embolectomy for acute limb ischemia, or vascular trauma. Administration of IL-10 may improve limb salvage and survival after significant torso or extremity ischemia.

The amount of IL-10 to be administered is preferably between 0.1 to 500 μg/kg of body weight, more preferably 1 to 50 μg/kg. The IL-10 may be of human or viral origin produced biologically from mammalian cellular sources or by recombinant DNA technology. Administration preferably takes place by intravenous, intramuscular or subcutaneous injection. The IL-10 is preferably administered from one to zero hours before the blood flow is reestablished.

In those surgical procedures in which temporary or sustained disruption of blood flow is anticipated to occur, as before surgical repair of thoracoabdominal or supraceliac aneursymal disease, or surgical procedures to the abdomen that will necessarily include the transient reduction in visceral blood flow, or for organ transplantation, the IL-10 is preferably given either as a single bolus injection one to zero hours before the ischemic event or as a continuous intravenous injection beginning one to zero hours before the ischemic event and extending during the perioperative period and continuing for at least eight hours after restoration of visceral blood flow.

For individuals in whom disrupted visceral blood flow has already occurred, as in those individuals with trauma or injury to the visceral organs or their blood supply, or in patients with systemic hypotension due to shock, the IL-10 would be preferably given either as a single bolus injection prior to or simultaneously with restoration of normal visceral blood flow or as a continuous intravenous injection prior to or simultaneously with restoration of normal visceral blood flow and extending for at least eight hours after restoration of visceral blood flow.

For individuals in whom disrupted skeletal blood flow has already occurred, as in those individuals with acute lower extremity ischemia due to embolic or thrombotic occlusion of peripheral blood vessels or acute ischemia due to vascular trauma, the IL-10 would be preferably given either as a single bolus injection prior to or simultaneously with restoration of normal blood flow or as a continuous intravenous injection prior to or simultaneously with restoration of

normal blood flow and extending for at least eight hours after restoration of blood flow.

Alternatively, the IL-10 may be administered by gene therapy or transfer using either liposomes and mammalian expression plasmids, mechanical delivery systems (gene gun) of viral transfection schemes, including but not limited to adenovirus, adeno-associated virus, retrovirus or herpes simplex virus constructs.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1(a), 1(b) and 1(c) illustrate the plasma TNF-α, IL-1β and IL-8 concentrations, respectively, following thoracoabdominal and infrarenal aortic aneurysm repair.

Figures 2(a), 2(b) and 2(c) illustrate the plasma IL-6, changes in plasma p55 concentrations, and changes in plasma p75 concentrations, respectively, following thoracoabdominal and infrarenal aortic aneurysm repair.

Figure 3 illustrates the changes in lung myeloperoxidase levels (neutrophil infiltration) in mice following supraceliac aortic cross clamp and treatment with inhibitors of TNF and IL-1.

Figure 4 illustrates the changes in lung permeability (^{125}I-albumin leakage) in mice following supraceliac aortic cross clamp and treatment with inhibitors of TNF and IL-1.

Figure 5, which illustrates the appearance of IL-10 in the circulation of mice following supraceliac aortic cross clamp used, shows plasma IL-10 concentrations in mice following supraceliac aortic cross-clamping and treatment with recombinant human IL-10.

Figure 6 illustrates the changes in lung myeloperoxidase levels (neutrophil infiltration) in mice following supraceliac aortic cross clamp and treatment with recombinant human IL-10.

DETAILED DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

As used herein, "interleukin-10" or "IL-10" is defined as a protein which (a) has an amino acid sequence of mature IL-10 (*e.g.*, lacking a secretory leader sequence) as disclosed in U.S. Patent No. 5,231,012 and (b) has biological activity that is common to native IL-10. Also included are muteins and other analogs, including the Epstein-Barr Virus protein BCRF1 (viral IL-10), which retain the biological activity of IL-10.

IL-10 suitable for use in the invention can be obtained from culture medium conditioned by activated cells secreting the protein, and purified by standard methods. Additionally, the IL-10, or active fragments thereof, can be chemically synthesized using standard techniques known in the art. See Merrifield, Science *233*:341 (1986) and Atherton *et al.*, *Solid Phase Peptide Synthesis: A Practical Approach*, 1989, I.R.L. Press, Oxford. See also U.S. Patent No. 5,231,012.

Preferably, the protein or polypeptide is obtained by recombinant techniques using isolated nucleic acid encoding the IL-10 polypeptide. General methods of molecular biology are described, *e.g.*, by Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor, New York, 2d ed., 1989, and by Ausubel *et al.*, (eds.) *Current Protocols in Molecular Biology*, Green/Woley, New York (1987 and periodic supplements). The appropriate sequences can be obtained using standard techniques from either genomic or cDNA libraries. Polymerase chain reaction (PCR) techniques can be used. See, *e.g.*, *PCR Protocols: A Guide to Methods and Applications*, 1990, Innis *et al.*, (Ed.), Academic Press, New York, New York.

Libraries are constructed from nucleic acid extracted from appropriate cells. See, *e.g.*, U.S. Patent No. 5,231,012, which discloses recombinant methods for making IL-10. Useful gene sequences can be found, *e.g.*, in various sequence databases, *e.g.*, GenBank and BMPL or nucleic acid and PIR and Swiss-Prot for protein, c/o Intelligenetics, Mountain View, California, or the Genetics Computer Group, University of Wisconsin Biotechnology Center, Madison, Wisconsin.

Clones comprising sequences that encode human IL-10 have been deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, under Accession Nos. 68191 and 68192. Identification of other clones harboring

the sequences encoding IL-10 is performed by either nucleic acid hybridization or immunological detection of the encoded protein, if an expression vector is used. Oligonucleotide probes based on the deposited sequences disclosed in U.S. Patent No. 5,231,012 are particularly useful. Oligonucleotide probes sequences can also be prepared from conserved regions of related genes in other species. Alternatively, degenerate probes based on the amino acid sequences of IL-10 can be used.

Standard methods can be used to produce transformed prokaryotic, mammalian, yeast or insect cell lines which express large quantities of the polypeptide. Exemplary *E. coli* strains suitable for both expression and cloning include W3110 (ATCC Bi, 27325), X1776 (ATCC No. 31244). X2282, and RR1 (ATCC Mp/ 31343). Exemplary mammalian cell lines include COS-7 cells, mouse L cells and CHP cells. See Sambrook (1989), *supra* and Ausubel *et al.*, 1987 supplements, *supra*.

Various expression vectors can be used to express DNA encoding IL-10. Conventional vectors used for expression of recombinant proteins in prokaryotic or eukaryotic cells may be used. Preferred vectors include the pcD vectors described by Okayama *et al.*, Mol. Cell. Biol. *3*:280 (1983); and Takebe *et al.*, Mol. Cell. Biol. *8*:466 (1988). Other SV40-based mammalian expression vectors include those disclosed in Kaufman *et al.*, Mol. Cell. Biol. *2*:1304 (1982) and U.S. Patent No. 4,675,285. These SV40-based vectors are particularly useful in COS-7 monkey cells (ATCC No. CRL 1651), as well as in other mammalian cells such as mouse L cells. See also, Pouwels *et al.*, (1989 and supplements) *Cloning Vectors: A Laboratory Manual*, Elsevier, New York.

The IL-10 may be produced in soluble form, such as a secreted product of transformed or transfected yeast, insect or mammalian cells. The peptides can then be purified by standard procedures that are known in the art. For example, purification steps could include ammonium sulfate precipitation, ion exchange chromatography, gel filtration, electrophoresis, affinity chromatography, and the like. See *Methods in Enzymology Purification Principles and Practices* (Springer-Verlag, New York, 1982).

Alternatively, IL-10 may be produced in insoluble form, such as aggregates or inclusion bodies. The IL-10 in such a form is purified by standard procedures that are well known in the art. Examples of purification steps include separating the inclusion bodies from disrupted host cells by centrifugation, and then

solubilizing the inclusion bodies with chaotropic agent and reducing agent so that the peptide assumes a biologically active conformation. For specifics of these procedures, see, *e.g.* Winkler *et al.*, Biochemistry *25*:4041 (1986), Winkler *et al.*, Bio/Technology *3*:9923 (1985); Koths *et al.*, and U.S. Patent No. 4,569,790.

The nucleotide sequences used to transfect the host cells can be modified using standard techniques to make IL-10 or fragments thereof with a variety of desired properties. Such modified IL-10 can vary from the naturally-occurring sequences at the primary structure level, *e.g.*, by amino acid, insertions, substitutions, deletions and fusions. These modifications can be used in a number of combinations to produce the final modified protein chain.

The amino acid sequence variants can be prepared with various objectives in mind, including increasing serum half-life, facilitating purification or preparation, improving therapeutic efficacy, and lessening the severity or occurrence of side effects during therapeutic use. The amino acid sequence variants are usually predetermined variants not found in nature, although others may be post-translational variants. Such variants can be used in this invention as long as they retain the biological activity of IL-10.

Modifications of the sequences encoding the polypeptides may be readily accomplished by a variety of techniques, such as site-directed mutagenesis (Gillman *et al.*, Gene *8*:81 (1987)). Most modifications are evaluated by routine screening in a suitable assay for the desired characteristics. For instance, U.S. Patent No. 5,231,012 describes a number of *in vitro* assays suitable for measuring IL-10 activity.

Preferably, human IL-10 is used for the treatment of humans, although viral IL-10 could possibly be used. Most preferably, the IL-10 used is recombinant human IL-10. The preparation of human IL-10 has been described in U.S. Patent No. 5,231,012. The cloning and expression of viral IL-10 (BCRF1 protein) from Epstein-Barr virus has been disclosed by Moore *et al.*, Science *248*:1230 (1990).

For examples of procedures and assays to determine IL-10 activity, see United States Patent No. 5,231,012. This patent also provides proteins having IL-10 activity and production of such proteins including recombinant and synthetic techniques.

To prepare pharmaceutical compositions of IL-10 for practice of this invention, the IL-10 is admixed with a pharmaceutically acceptable carrier or

excipient which is preferably inert. A pharmaceutical carrier can be any compatible non-toxic substance suitable for delivery of the polypeptide to a patient. Preparation of such pharmaceutical compositions is known in the art; see, *e.g.*, *Remington's Pharmaceutical Sciences, and U.S. Pharmacopeia: National Formulary*, Mack Publishing Company, Easton, PA (1984).

Compositions may be ingested orally or injected into the body. Formulations for oral use include compounds to protect the polypeptides from proteases which occur in the gastrointestinal tract. Injections are usually intramuscular, subcutaneous, intradermal or intravenous. Alternatively, intra-articular injection or other routes could be used in appropriate circumstances.

When administered parenterally, the compositions can be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutical carrier. For instance, the IL-10 may be administered in aqueous vehicles such as water, saline or buffered vehicles with or without various additives and/or diluting agents. Examples of suitable carriers are normal saline, Ringer's solution, dextrose solution, and Hank's solution. Non-aqueous carriers such as fixed oils and ethyl oleate may also be used. A preferred carrier is 5% dextrose/saline. The carrier may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, *e.g.*, buffers and preservatives. However, the IL-10 in the composition is preferably formulated in purified form substantially free of aggregates and other proteins. In addition, it should be noted that a suspension, such as a zinc suspension, can be prepared to include the polypeptide. Such a suspension can be useful for subcutaneous (SQ) or intramuscular (IM) injection.

It is believed that ischemia-reperfusion injury is caused, at least in part, by the release of excess amounts of proinflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-8. Examples 1 and 2 were performed to test this theory and the effect IL-10 has on visceral ischemia-reperfusion injury. Example 3 sets forth the application of the invention to a human patient undergoing aortic aneurysm repair. Example 4 was performed to demonstrate that IL-10 will attenuate the skeletal muscle and pulmonary injury after hindlimb ischemia-reperfusion in a rat model.

EXAMPLE 1

Initial studies investigated prospectively the associative relationship between the proinflammatory cytokine response and morbidity and mortality

following visceral ischemia and reperfusion in humans by measuring proinflammatory cytokine levels in patients undergoing thoracoabdominal or infrarenal aortic aneurysm repair, and comparing these results to the incidence of postoperative organ dysfunction.

Sixteen human patients undergoing elective repair of a thoracoabdominal aortic aneurysm and 9 patients undergoing elective infrarenal aortic aneurysm repair agreed to arterial blood sampling for proinflammatory cytokine measurements. Each thoracoabdominal aortic aneurysm was repaired through a left flank incision using a retroperitoneal approach. The diaphragm was divided circumferentially, allowing exposure of the descending thoracic aorta. Prior to cross-clamping, each patient was given mannitol (0.5 gm/kg) and solumedrol (15 mg/kg). Depending upon the location of the aneurysm, the visceral arteries were sewn onto the graft as a Carrel patch or as part of the proximal anastomosis with an extensive posterior taper to the graft. Once the repair was completed, coagulation products (platelets and fresh frozen plasma) were infused as needed. Preoperatively, a catheter was placed in the lumbar spinal column and cerebrospinal fluid drained to maintain intrathecal pressure at 5-10 cm water. Infrarenal abdominal aortic aneurysms were repaired transperitoneally using standard surgical techniques and the aorta was reconstructed using either a straight tube graft to the aortic bifurcation or a bifurcated graft to the internal/external iliac artery bifurcation.

In both groups of patients, arterial blood samples (7 ml) were obtained following induction of anesthesia, just prior to aortic cross-clamp placement, just prior to clamp release, and at timed intervals (1, 2, 4, 6 to 8, 24 hrs and daily for 7 days) after reperfusion. Clinical and laboratory data were collected prospectively from all patients to determine preoperative risk factors and postoperative organ dysfunction patterns. Data collected included operative parameters (total operative time, aortic cross-clamp time, estimated blood loss, intraoperative complications), postoperative course (complications, organ dysfunction) and causes of death. Laboratory values were analyzed during the initial 7 postoperative days to focus on the injury associated with tissue ischemia-reperfusion after thoracoabdominal and infrarenal aortic aneurysm repair.

Postoperative pulmonary dysfunction was defined as the need for positive-pressure mechanical ventilatory assistance for greater than 7 days while postoperative hepatic dysfunction was defined as peak lactate dehydrogenase

(LDH) levels greater than 500 U/L and either serum transaminase levels (AST/ALT) greater than 200 U/L or an increase in total bilirubin levels greater than 3 mg/dl. Renal dysfunction was defined as an increase in serum creatinine of 2 mg/dl or more over preoperative baseline, while a platelet count less than 50,000/mm^3 or a drop in white blood cell count below 4,500 /mm^3 indicated the presence of hematopoietic dysfunction. Patients with 2 or more organ systems meeting these criteria were designated as having multiple system organ dysfunction [MSOD].

Freshly thawed plasma samples were assayed for TNF-α, IL-1, IL-6, IL-8 and TNF-α shed receptors (p55 and p75) by ELISA. The sensitivity of the TNF-a, IL-1, IL-6, IL-8, p55 and p75 assays are 14, 10, 27, 313, 14 and 17 pg/ml, respectively.

The mortality and morbidity data from the 16 patients undergoing thoracoabdominal aortic aneurysm repair and the 9 patients undergoing infrarenal aortic aneurysm repair are reported in Table I.

TABLE 1

Incidence of organ dysfunction following thoracoabdominal or infrarenal aortic aneurysm repair.

Data presented shows that the frequency of pulmonary dysfunction and MSOD following thoracoabdominal aortic aneurysm repair was significantly higher than following abdominal aortic aneurysm repair.

	Thoracoabdominal Aortic Aneurysm (n=16)	Infrarenal Aortic Aneurysm (n=9)
Mortality	19%	0%
Pulmonary Dysfunction	56%*	11%
Tracheostomy	25%	0%
Renal Dysfunction	38% **	0%
Dialysis	13%	0%
Hepatic Dysfunction	31%	0%
Hematopoietic Dysfunction	38% **	0%
Leukopenia	13%	0%
MSOD	44% *	0%

* p<0.05 by Fisher's exact test
** p=0.057 by Fisher's exact test

Three patients died after thoracoabdominal aortic aneurysm repair, 2 from MSOD and 1 from cardiac arrest. Pulmonary dysfunction occurred in 9 patients

and placement of a temporary tracheostomy was eventually required in 4 patients. Renal dysfunction developed in 6 patients and hemodialysis was necessary in 2 of them. Hepatic dysfunction, thrombocytopenia, and leukopenia developed after thoracoabdominal aortic aneurysm repair in 5, 6, and 2 patients, respectively, and lower extremity dysfunction due to spinal cord injury occurred in 2 patients. In contrast, there were no operative deaths after infrarenal aortic aneurysm repair (Table 1). Pulmonary dysfunction occurred in only 1 patient and there was no evidence of renal, hepatic, hematopoietic or lower extremity dysfunction in any patient.

The peak plasma cytokine responses in both groups of patients are reported in Table 2.

TABLE 2

Peak proinflammatory cytokine concentrations following thoracoabdominal or infrarenal aortic aneurysm repair.

Plasma samples were obtained 0, 1, 2, 4, 6-8, 24, 48, 72 hours and daily for up to seven days following thoracoabdominal or infrarenal aortic aneurysm repair. Peak concentrations are reported here. Levels of all proinflammatory cytokines were significantly higher in patients following thoracoabdominal than infrarenal aortic aneurysm repair ($p<0.05$).

	Thoracoabdominal Aortic Aneurysm (n=16)	Infrarenal Aortic Aneurysm (n=9)
TNF-α pgs/ml	161±58	10±10
IL-1b pgs/ml	133±59	24±10
IL-6, pgs/ml	1,280±664	181±108
IL-8, pgs/ml	410±139	137±77
p55, change from baseline in pgs/ml	751±668	204±218
p75, change from baseline in pgs/ml	5,201±1,983	383±171
C3a, µg/ml	111±21	30±7

all values are significantly different between the two groups, by two-way ANOVA, $p<0.05$

Plasma TNF-α IL-1, IL-6 and IL-8 concentrations were undetectable prior to surgery. Following surgical repair of thoracoabdominal aortic aneurysms, a monophasic TNF-α response was detected in 11 of 16 patients (69%) (Figures 1(a), 1(b) and 1(c)). TNF-α levels peaked 4 hours after reperfusion and then gradually decreased toward baseline over the next 24 hours. IL-6 and IL-8 levels also increased in a monophasic pattern with peak levels again occurring 4 hours

after reperfusion in 16 (100%) and 11 (70%) patients, respectively; however, unlike the pattern seen with TNF-α, circulating IL-6 and IL-8 levels decreased to baseline within 8 hours. IL-1 was also detected in a monophasic pattern in 50% of the thoracoabdominal aortic aneurysm patients, but its peak levels occurred at 1 hour after reperfusion and IL-1 levels returned to baseline levels 4-6 hours after reperfusion. The plasma concentrations of the soluble TNF-α receptors, p55 and p75, were increased after thoracoabdominal aortic aneurysm repair in 12 (75%) and 16 (100%) of the patients assayed, respectively (Figure 2). p55 receptor concentrations reached a zenith at 24 hours and remained elevated for several days while p75 receptor concentrations continued to increase throughout the initial 48 hours after reperfusion. In contrast to thoracoabdominal aortic aneurysm repair patients, peak serum levels of TNF-α, IL-1, IL-6, IL-8, p55 and p75 were 3 to 15-fold less in patients undergoing infrarenal abdominal aortic aneurysm repair (Table 2 and Figures 1(a), 1(b), 1(c) and 2(a), 2(b) and 2(c)).

A retrospective analysis was performed in an effort to establish an associative relationship between patient clinical outcome and the concentrations of various proinflammatory cytokines. Patients undergoing thoracoabdominal aortic aneurysm repair in whom peak TNF-α levels were less than 150 pg/ml did not experience single or multiple organ dysfunction, while single organ dysfunction and MSOD were common in patients whose peak TNF-α levels were greater than 150 pg/ml (Table 3).

TABLE 3

Relationship between post-operative organ dysfunction and peak circulating TNF-α levels.

	TNF-α <150 pgs/ml	TNF-α >150 pgs/ml
Mortality	1 death cardiac	2 deaths - MSOD
Pulmonary Dysfunction	0%	57%**
Renal Dysfunction	0%	71% *
Dialysis	0%	29%
Hepatic Dysfunction	0%	71% *
Hematopoietic Dysfunction	0%	71% *
Leukopenia	0%	28%
MSOD	0%	86% *

* p<0.05 by Fisher's exact test

**p=0.07 by Fisher's exact test

In addition, patients who developed MSOD after thoracoabdominal aortic aneurysm repair had higher circulating levels of all assayed cytokine and soluble TNF-α receptors (p55 and p75) as compared to patients without MSOD (Table 4); however, only TNF-α and p55 receptor levels were statistically different ($p<0.05$) while there was a trend toward higher levels of IL-1, IL-6, IL-8 and p75 receptors in patients who developed MSOD as compared to patients without MSOD (Table 4).

TABLE 4

Plasma proinflammatory cytokine concentrations in patients with and without evidence of multisystem organ dysfunction (MSOD).

Peak plasma concentrations of TNF, IL-6, p55 and p75 were significantly higher in patients following thoracoabdominal aortic aneurysm repair with MSOD than in patients either following thoracoabdominal aortic aneurysm repair without MSOD or in patients following infrarenal aortic aneurysm repair.

	Thoracoabdominal Aortic Repair with MSOD	Thoracoabdominal Aortic Repair w/o MSOD	Infrarenal Aortic Repair
cross-clamp time	56±5 mins	33±4 mins	nr
TNFα	414±59*	86±55	10±10
IL-1β	173±112	102±62	24±10
IL-6	4,907±1887*	344±66	181±108
IL-8	601±259	376±107	137±77
p55	+3,515±711*	+452±415	+204±218
p75	+9,469±1940*	+4,136±1,884	+382±171

values for p55 and p75 are changes from baseline. All values are in pgs/ml.
* $p<0.05$ versus no MSOD by 2-way ANOVA

nr = not reported

The results presented here demonstrate that surgical repair of thoracoabdominal aortic aneurysms which causes visceral ischemia-reperfusion injury results in a systemic proinflammatory cytokine response characterized by the appearance of TNF-α, IL-1, IL-6 and IL-8 in the blood as early as 1 to 4 hours after release of the cross-clamp. Additionally, the presence and magnitude of this proinflammatory cytokine response is associated with the incidence of postoperative organ dysfunction after thoracoabdominal aortic aneurysm repair. Ischemia and subsequent reperfusion injury of the viscera appear to be critical for the induction of this systemic proinflammatory cytokine response, because the magnitude of the proinflammatory cytokine response is 3 to 15-fold less in patients undergoing repair of the infrarenal aorta where visceral ischemia/reperfusion does

not occur than following thoracoabdominal aortic repair. In addition, patients having infrarenal aortic aneurysm repair, in whom visceral ischemia is avoided, have a significantly lower incidence of postoperative organ dysfunction.

To further explore the direct role of acute visceral ischemia in mediating this proinflammatory cytokine response and associated organ dysfunction, an additional 8 patients were studied following elective thoracoabdominal aortic aneurysm repair. However, in this case, duration of visceral ischemia was reduced by left atrial-femoral artery bypass (LAFBP) and retrograde perfusion of the visceral arteries. LAFBP provides distal blood flow during repair of thoracoabdominal aneurysms and reduces visceral ischemia time. We prospectively examined the effect of LAFBP on patients undergoing thoracoabdominal aortic repair (n=8) and compared the cytokine response, morbidity, and mortality to patients undergoing standard thoracoabdominal aortic aneurysm repair (n=16) without the benefit of LAFBP.

Timed measurement of cytokine levels was done during the 48 hour perioperative period and cytokine levels were measured by ELISA. Clinical data concerning postoperative pulmonary, hepatic, renal, and hematopoietic dysfunction were also prospectively collected. Patients undergoing repair of thoracoabdominal aortic aneurysms with LAFBP had shorter visceral ischemia times (18± 5 min. vs 45±12 min.) and statistically significant reductions in circulating TNF-α, IL-10, and p75 levels ($p<0.05$ by two-way ANOVA) when compared to the control group (Table 5).

TABLE 5

Plasma proinflammatory cytokine concentrations in patients undergoing thoracoabdominal aortic aneurysm with left atrial femoral bypass (LAFB) or without LAFB

Peak plasma concentrations of TNF-α, IL-10 and p75 were significantly higher in patients following thoracoabdominal aortic aneurysm repair without LAFB than in patients following thoracoabdominal aortic aneurysm repair with LAFB.

	Repair with LAFBP(n+8)	Repair W/O LAFBP (n=16)
TNF-α, pgs/ml	10±10	161±58*
IL-6, pgs/ml	2320±1644	1280±664

IL-8, pgs/ml	410±139	458±402
IL-10, pgs/ml	60±29	806±260*
p55, baseline pgs/ml	+741±298	+751±668
p75, baseline pgs/ml	+641±406	+5201±1983*

* $p<0.05$

Additionally, the incidence of pulmonary dysfunction, renal dysfunction, thrombocytopenia, multisystem organ dysfunction, and mortality were reduced in patients undergoing LAFBP, although the numbers were too small to show any statistical difference.

These findings suggest that acute visceral ischemia-reperfusion injury secondary to thoracoabdominal aortic aneurysm repair is associated with a high rate of morbidity and multisystem organ dysfunction that is not seen with similar surgical procedures that do not cause visceral ischemia. Furthermore, techniques aimed at reducing the duration of ischemia during aortic cross-clamping (left atrial-femoral bypass) appear to reduce the magnitude of the TNF-α and IL-1 responses.

EXAMPLE 2

Experiments in mice have been conducted that demonstrate that pretreatment with recombinant human IL-10 can reduce distant organ injury in a clinically relevant model of acute visceral ischemia-reperfusion injury. The initial goal of these studies was to develop a clinically relevant model of acute ischemia-reperfusion injury that demonstrated evidence of organ injury that was dependent upon an endogenous proinflammatory cytokine response that could be inhibited by either a TNF-α receptor construct or a monoclonal antibody against the IL-1 type I (p80) receptor (35F5, Hoffmann-LaRoche, Nutley, NJ).

Thirty mice (C57BL/6, approx. 20 gm) were anesthetized with pentobarbital. In 16 of these animals, the supraceliac aorta was cross-clamped for 30 minutes. Six animals had their infrarenal aorta cross-clamped for 30 minutes, while another 8 animals received only anesthesia, incision and bowel mobilization without aortic cross-clamping. Two hours prior to supraceliac aortic cross-clamping, 8 of the 16 animals were pretreated with the intraperitoneal injection of 10 mg/kg BW of TNF-

bp (a TNF-α binding protein that is comprised of the extracellular domains of two p55 TNF-α receptors covalently linked to polyethylene glycol). Two hours after aortic clamp removal and abdominal wound closure, the animals were sacrificed and lung neutrophil infiltration was evaluated by MPO content. Results are shown in Figure 3. Supraceliac aortic cross-clamping resulted in a significant increase in pulmonary neutrophil infiltration at 2 hours, which was not seen in animals that had the infrarenal aorta cross-clamped. Pretreatment of the animals undergoing supraceliac aortic cross-clamping with TNF-bp significantly attenuated this increase.

To determine the effect of visceral ischemia-reperfusion on lung capillary function, 50 mice were anesthetized with pentobarbital, and in 34 animals the supraceliac aorta was cross-clamped for 30 minutes. Eleven of these animals were pretreated with TNF-bp (10 mg/kg) while 9 were pretreated with 150 μg of a monoclonal antibody directed against the murine IL-1 receptor type I (35F5). It has been previously reported that this antibody blocks IL-1 binding to the functional IL-1 type I receptor and attenuates IL-1-mediated inflammation. Control groups consisted of a sham operation group (n=10) and an infrarenal cross-clamp group (n=6). After the removal of the aortic cross-clamps and the onset of reperfusion, the animals were injected with 1 μCi of I^{125} labeled albumin i.v. via the inferior vena cava. At the end of 4 hours of reperfusion the animals were sacrificed and the lungs were treated with bronchoalveolar lavage (BAL) with 1.75 ml of normal saline. The pulmonary mean permeability index was calculated as the ratio of CPM/gm BAL over CPM/gm blood. The results are shown in Figure 4. Both pretreatment with TNF-bp and 35F5 decreased pulmonary capillary injury ($p < 0.05$), with 35F5 having a more pronounced effect.

Thus, these findings demonstrate that the lung injury secondary to supraceliac cross-clamping in the mouse is a result of endogenous production of TNF-α or IL-1. Inhibiting either of these cytokines with novel inhibitors of either TNF-α or the IL-1 type I receptor can minimize the lung injury secondary to visceral ischemia-reperfusion injury.

To demonstrate that similar effects can be obtained by immediate pretreatment with recombinant human IL-10, an additional study was conducted in mice subjected to supraceliac aortic cross-clamping. Visceral ischemia was induced in 90 female C57BL/6 mice (20-22gm) by supraceliac aortic cross-clamping for 25-30 minutes. An additional 38 mice received sham procedures.

Plasma IL-10 levels were measured by ELISA at 1, 2, 4 and 8 hrs after reperfusion, and lung neutrophil infiltration was determined by MPO assay at 2 hrs, as previous studies had revealed that maximal neutrophil infiltration occurred in the lung at 2 hrs. Thirty-six of the mice undergoing visceral ischemia-reperfusion were pretreated with 0.2μg (n=7), 2μg (n=13), 5μg (n=6), or 20μg (n=10) of recombinant human IL-10.

Mean plasma IL-10 concentrations peaked at 9,120 pg/ml 2 hours following 25-30 minutes of supraceliac aortic cross-clamping (Figure 5). Visceral ischemia-reperfusion injury also resulted in an 6-fold increase in lung neutrophil infiltration ($p<0.05$) (Figure 6). When mice were pretreated with exogenous IL-10, neutrophil infiltration was significantly reduced ($p<0.05$ for all doses). Maximal improvements in pulmonary neutrophil infiltration were attained with 5 μg/mouse (250 μg/kg BW) of IL-10.

Visceral ischemia-reperfusion injury associated with supraceliac aortic cross-clamping promotes the release of IL-10, while exogenous IL-10 administration prior to aortic cross-clamping limits pulmonary injury in this model of acute visceral ischemia-reperfusion injury. Thus, exogenous IL-10 may offer a novel therapeutic approach to decrease complications associated with thoracoabdominal aortic aneurysm repair and other ischemia-reperfusion injuries.

Hypothetical Example 3 illustrates a preferred application of the invention contemplated for treating humans.

EXAMPLE 3

A 58 year-old white male presents to the emergency room of a local University hospital complaining of several months of intermittent sharp epigastric and periumbilical abdominal pain, with no other significant symptoms. The patient has no history of any significant medical problems other than a history of atherosclerotic disease. On physical exam, the patient is found to have a nontender, pulsatile mid-abdominal mass, with an audible bruit. Laboratory examination including hematology, biochemistries, liver function tests, urinalysis and amylase are all within normal limits. Flat and upright abdominal x-rays, as well as chest x-rays, are unremarkable. An abdominal CT scan with cuts through the lower chest reveals an aortic aneurysm extending from the level of the diaphragmatic hiatus to the aortic bifurcation, 6.5 cm in largest diameter.

After informed consent is obtained, the patient is prepared for surgery. One hour prior to skin incision, the patient is given a single bolus administration of recombinant human IL-10 at a dose of 10μg/kg body weight through an indwelling catheter in the median cubital vein. In addition, a lumbar catheter is placed to drain cerebrospinal fluid to maintain intrathecal pressure at 5-10 cm water pressure. Under a general inhalation anesthetic, a left flank incision is made, gaining access to the aorta via a retroperitoneal approach. The diaphragm is divided circumferentially to allow exposure of the thoracic aorta. After the patient is given intravenous doses of mannitol (0.5 gm/kg) and solumedrol (15 mg/kg), the aorta is cross-clamped proximal to the cephalad aspect of the aneurysm and distal to the aortic bifurcation at the level of the proximal external iliac arteries. The aorta is then reconstructed utilizing a bifurcated graft from the level of the caudal thoracic aorta to the external iliac arteries bilaterally. The celiac and superior mesenteric arteries are then sewn to the graft as a Carrel patch. Cross-clamp time and period of warm visceral ischemia is 42 minutes. The aortic cross-clamps are thereafter removed, restoring perfusion of the viscera, pelvis, and lower extremities. Three units of packed red blood cells and two units of fresh frozen plasma are infused. Incisions are then closed, and the patient is transported to the surgical intensive care unit intubated and receiving ventilatory assistance, but hemodynamically stable. After an unremarkable night, the patient is extubated on post-operative day 1. On post-operative day 2, the patient is transferred out of the intensive care unit to the surgical ward. The patient has return of bowel function on post-operative day 5, and is discharged home, ambulating without difficulty, tolerating a regular diet, with his incision healing nicely, with no evidence of infection on post-operative day 7.

Another preferred application of this invention is administration of IL-10 to a patient one to zero hours before the patient receives a major organ transplant. This invention is especially applicable to treatment of ischemia-reperfusion occurring in the visceral section of the body. Regardless of which procedure causes or is expected to cause the ischemia- reperfusion injury, the inventive method of treatment will be deemed successful if one or more of the signs or symptoms of ischemia-reperfusion injury are alleviated or fail to appear at all.

EXAMPLE 4

The following experiments in rats demonstrate that pretreatment with exogenous human IL-10 may decrease lung and soleus muscle injury in a clinically relevant model of hindlimb ischemia-reperfusion injury.

Twenty eight male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA., approx. 350 gm) were anesthetized with pentobarbital intraperitoneally (40 mg/kg, Abbott Laboratories, Chicago, IL.). In twenty of the rats, bilateral hindlimb ischemia was produced by placement of a rubber band tourniquet across the upper thigh of both lower extremities. The cessation of arterial blood flow was confirmed by the absence of a Doppler signal in the superficial femoral artery. The remaining eight rats received anesthesia alone.

Half of the animals in each group (10 in the ischemic group and 4 of the non-ischemic controls) were pretreated with 10 µg of recombinant IL-10. After the induction of anesthesia, a catheter was placed into the right atrium through the external jugular vein for blood sampling and infusion of normal saline (1 cc/hr). Recombinant human IL-10 (rhIL-10, 10 µg approx. 30 µg/kg BW IV) or a comparable volume of normal saline was administered twenty minutes prior to the onset of ischemia or at comparable times for the non-ischemic controls.

After 4 hours of ischemia, the tourniquets were removed and the extremity was reperfused. The restoration of arterial blood flow was confirmed by the presence of a Doppler signal in the superficial femoral artery. Blood (0.5 cc) was sampled at the time of central venous line placement, at reperfusion, 30 minutes after reperfusion, 60 minutes after reperfusion, and hourly thereafter. Blood was sampled at comparable time periods in the non-ischemic controls.

The animals were euthanized (pentobarbitol 100 mg/kg BW IV) after 4 hours of reperfusion or at comparable times for the non-ischemic controls. The soleus muscle from one hindlimb and one lung were analyzed for assessment of neutrophil infiltration. Soleus muscle and pulmonary neutrophil sequestration were quantified by the tissue myeloperoxidase (MPO) levels (Warren *et al.*, 1989, J.Clin.Invest. 84:1873).

The remaining soleus muscle and lung tissue were analyzed to quantify the capillary and/or cellular injury. Skeletal muscle and lung capillary endothelial cell

injury were quantified by uptake of I^{125} labeled albumin (Welbourn *et al.*, 1991, J. Appl. Physiol. 70:2645). Skeletal muscle cellular injury was quantified by the uptake of Tc^{99} labeled pyrophosphate (Blebea *et al.*, 1988, J. Vasc. Surg. 8:117). The mean capillary permeability index (CPI) and the skeletal muscle injury index (SMII) were calculated using the following formulas:

CPI = (I^{125} muscle/muscle mass) / (I^{125} blood/blood mass).
SMII = (Tc^{99} muscle/muscle mass) / (Tc^{99} blood/blood mass).

Circulating bioactive TNF was measured using the TNF-sensitive WEHI murine fibrosarcoma cell line (Van Zeed *et al.*, 1992, PNAS 89:4845).

Skeletal Muscle Injury:

The results are shown in Table 6. The hindlimb I/R resulted in significant skeletal muscle injury. Both the mean soleus muscle capillary permeability index (MCPI) and the mean soleus skeletal muscle injury index (SMII) after hindlimb I/R were significantly greater than the non-ischemic controls. Pretreatment of the animals with recombinant human IL-10 prior to hindlimb ischemia resulted in a significantly lower skeletal muscle capillary injury that was not significantly different from the non-ischemic control. Pretreatment with human IL-10 prior to ischemia also resulted in a decrease of the skeletal muscle cellular injury, although the difference did not reach significance. However, again the skeletal muscle cellular injury in the ischemic animals pretreated with human recombinant IL-10 was not different than the non-ischemic controls. Neutrophil infiltration in the skeletal muscle was not detected by the MPO assay for any of the 4 treatment groups.

Table 6

Skeletal Muscle Injury

	Muscle Capillary Permeability Index	Skeletal Muscle Injury Index
I/R	1.51 ± 1.46	0.94 ± 0.83
I/R + IL-10	0.39 ± 0.38*	0.36 ± 0.51
SHAM	0.03 ± 0.01*	0.05 ± 0.02
SHAM + IL-10	0.19 ± 0.23*	0.26 ± 0.30

* significantly different from I/R (ANOVA, Duncan's multiple range test; $p<.05$)

Lung Injury

The results are shown in Table 7. The hindlimb ischemia-reperfusion also resulted in significant pulmonary vascular injury as determined by the leakage of I^{125} albumin into the lungs. Both the mean pulmonary capillary permeability index and the mean pulmonary neutrophil infiltration in the animals subjected to hindlimb ischemia-reperfusion were significantly greater than the non-ischemic controls. Pretreatment with human recombinant IL-10 significantly reduced the lung capillary injury after hindlimb ischemia-reperfusion and the PCPI values in the pretreated animals were not different from the non-ischemic controls. In contrast, pretreatment with human recombinant IL-10 resulted in a significant increase in the lung myeloperoxidase content after hindlimb ischemia-reperfusion. Although a ready explanation for this latter finding is not immediately forthcoming and it is in no way essential to this invention, it may well have been that IL-10 prevented the activation and degranulation of neutrophils in the lung. In this model, IL-10 may not have prevented the recruitment of neutrophils into the lung, but prevented the degranulation of their toxic contents, thus explaining both the higher MPO levels and reduced endothelial injury. Treatment of the non-ischemic controls with human recombinant IL-10 also increased the pulmonary neutrophil infiltration, although this difference was not significant.

Table 7
Lung Injury

	Lung Capillary Permeability Index	Lung Myeloperoxidase Levels (U/g)
I/R	2.32 ± 1.25	19.54 ± 11.07
I/R + IL-10	1.02 ± 1.22*	37.06 ± 4.35*
SHAM	0.65 ± 0.22*	7.82 ± 3.03*#
SHAM + IL-10	0.44 ± 0.43*	11.02 ± 2.01*#

*significantly different from I/R (ANOVA, Duncan's; $p<.05$)
#significantly different from I/R + IL-10 (ANOVA, Duncan's multiple range test; $p<.05$)

TNF Assay: Serum was assessed for circulating TNF in 6/10 rats undergoing ischemia-reperfusion and TNF levels ≥ 50 pg/ml were detected in 67% (4/6). In contrast, significant circulating TNF levels were found in only 30% (3/10) of the ischemic animals pretreated with human recombinant IL-10. Serum TNF levels of ≥ 50 pg/ml were not detected in any of the non-ischemic control animals.

These findings demonstrate that the anti-inflammatory cytokine IL-10 attenuates both local and distant organ injury resulting from hindlimb ischemia-reperfusion. The findings therefore provide indirect evidence that the associated injuries are mediated in part by the proinflammatory cytokines and are amenable to IL-10 based treatments.

* C L M S *

THIS SHEET HAS BEEN REPLACED

CLAIMS

1. The use of IL-10 for the manufacture of a medicament for treating or preventing ischemia-reperfusion injury.

2. A method for the manufacture of a pharmaceutical composition for treating or preventing ischemia-reperfusion injury, comprising admixing a pharmaceutically acceptable carrier and an effective amount of IL-10.

3. The use or method of claim 1 or 2 wherein the ischemia-reperfusion injury is caused by a major organ transplant or repair of an aneurysm.

4. The use or method of claim 1 or 2 wherein the ischemia-reperfusion injury is caused by surgical repair of a thoracic aortic aneurysm, a suprarenal aortic aneurysm, liver, kidney, small intestine, or pancreas transplant, hepatic and biliary surgical resections, total or partial pancreatectomy, total and partial gastrectomy, esophagectomy, colorectal surgery, vascular surgery for mesenteric vascular disease, abdominal insufflation during laparoscopic surgical procedures, blunt or penetrating trauma to the abdomen including gun shot wounds, stab wounds or penetrating wounds or blunt abdominal trauma secondary to deacceleration injury and/or motor vehicle accidents, hemorrhagic shock due to blood loss, cardiogenic shock to myocardial infarction or cardiac failure, neurogenic shock or anaphylaxis.

5. The use or method of claim 1 or 2 wherein the ischemia-reperfusion injury is caused by surgical repair of an abdominal aortic aneurysm, aortic femoral, popliteal or tibeal bypass for claudication or limb threatening ischemia, repair of popliteal or femoral aneurysms, bypass, thrombectomy or embolectomy for acute limb ischemia, or vascular trauma.

6. The method or use of any of claims 1-5 wherein the IL- 10 is human or viral IL-10.

This Sheet Has Been Replaced