Local and Remote Ischemia-Reperfusion Injury Is Mitigated in Mice Overexpressing Human C1 Inhibitor

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Key Words

Ischemia-reperfusion injury · Complement · C1 inhibitor · Transgenic mouse · Endothelial disruption · Permeability index

Abstract

Activation of the classical complement pathway is crucially involved in complement-mediated endothelial cell damage in ischemia-reperfusion injury. C1 inhibitor is the only known physiological inhibitor of classical complement pathway activation. Transgenic mice overexpressing human C1 inhibitor were used in a surgical lower torso and a liver ischemia-reperfusion model. Organ-specific endothelial disruption was determined by ¹²⁵I-tagged albumin extravasation. In the lower torso ischemia-reperfusion model, transgenic mice overexpressing the C1 inhibitor were protected in the muscle and the lungs from endothelial cell damage. In the liver ischemia-reperfusion model, endothelial cell integrity was preserved in transgenic animals in the liver, the gut and the lungs. Our data indicate that inhibiting complement activation by a transgenic approach is effective in protection against ischemia-reperfusion injury.

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Introduction

Tissue damage due to ischemia-reperfusion injury is of crucial clinical importance in organ transplantation as well as in a variety of disorders like hemorrhagic shock, myocardial infarction, stroke, and embolic disease [1–4]. The pathophysiological key events in ischemia-reperfusion injury have been characterized over the past 30 years as complement activation and neutrophil stimulation [5, 6]. The complement system is activated during ischemia-reperfusion through three different pathways, the classical, the alternative, and the lectin pathway, which results in the formation of the membrane attack complex (C5b-9) and ultimately endothelial cell disruption. During complement activation anaphylatoxins like C5a and C3a cause chemotaxis of leukocytes, degranulation of phagocytic cells, and a further increase of vascular permeability [7, 8].

An attractive approach to modify complement activation consists in the application of the natural complement regulator C1 inhibitor. C1 inhibitor is the only known physiological inhibitor of the classical complement cascade activation [9]. It belongs to the superfamily of serine protease inhibitors and is a heavily glycosylated serum protein of 105 kD [10, 11]. It inhibits the antibody-depen-

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0014-312X/04/0363-0142\$21.00/0

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dent formation of C1 by steric interaction with the C1-subunits C1r and C1s. Furthermore, it interferes with the lectin pathway by inhibition of the serum mannose-binding lectin-associated proteases [12]. Additional proteases inactivated by C1 inhibitor include activated coagulation factors XI and XII, kallikrein, plasmin, and tissue-type plasminogen activator.

The classical complement pathway cascade due to natural-antibody-mediated C1 activation is important in ischemia-reperfusion injury [13–15] and C1 inhibitor can effectively control this activation. In normal serum C1 inhibitor is present in a 7-fold molar excess of C1. Additional increase to more than 10-fold molar excess of C1 inhibitor blocks C activation completely [9].

The known minor variations of C1 inhibitor between different species [16] do not impede the functionality of the protein as shown by the successful use of the human C1 inhibitor in feline [17, 18] canine [19] and rodent [20, 21] animal models. Complement inhibition in the mouse by human C1 inhibitor was demonstrated in vitro [22] and recently in vivo in a study of lower torso ischemia-reperfusion injury in wild-type mice [21].

Due to the proven protective in vivo effect of human C1 inhibitor in ischemia-reperfusion experimentation [21] but also because of the short half-life of administered C1 inhibitor [16], it seemed attractive to test the capacity of human C1 inhibitor overexpressing transgenic mice [11] to resist complement-mediated endothelial cell disruption in ischemia-reperfusion injury. Using the well-established method of intravenous ¹²⁵I-labelled bovine albumin injection, the integrity of tissue-specific endothelium is assessed by the use of the permeability index, and a destroyed vascular endothelium is characterized by elevated permeability index values due to extravasation of tagged albumin [2, 23, 24].

Tissue-specific radioactivity was compared between three experimental groups: Transgenic, human C1 inhibitor overexpressing mice with ischemia-reperfusion injury, wild-type control animals with ischemia-reperfusion injury, and sham-operated control mice in a surgical lower torso ischemia-reperfusion model and a liver ischemia-reperfusion model.

Material and Methods

Human C1 Inhibitor Overexpressing Transgenic Mice

All animal experimentation was approved by the local committee for animal welfare in accordance with the European Convention on Animal Care.

Transgenic human C1 inhibitor overexpressing mice were generated as described and were kindly prepared by G. Vinci, Institut Pasteur, Paris, France [11]. Only animals with high plasma expression of human C1 inhibitor (i.e. 1–2 mg/ml) were used for ischemia-reperfusion experimentation.

Anesthesia

Adult SPF C57B/6 mice and transgenic human C1 inhibitor overexpressing mice, 25–32 g, were kept under standard conditions for laboratory animals with a 12-hour light/dark cycle. They were fed standard chow and given water ad libitum. Operative procedures were performed under general intraperitoneal (i.p.) anesthesia with an initial combination of ketamine (0.065 mg/g body weight; Ketalar®, Parke Davis, Baar, Switzerland), xylazine (0.013 mg/g body weight; Rompun®, Bayer AG, Leverkusen, Germany) and acepromazine (0.002 mg/g body weight; Sedalin®, Chassot GmbH, Ravensburg, Germany). Anesthesia was then maintained by i.p. application of 1 mg ketamine, 0.2 mg of xylazine and 0.03 mg of acepromazine using an i.p. catheter through the right lateral abdominal wall every 90 min. Ringer's solution was administered (0.2 ml) i.p. every hour.

Surgical Procedures

Lower Torso Ischemia-Reperfusion

Animals were immobilized in a supine position and the abdomen entered through a midline incision. The infrarenal aorta and vena cava were dissected free and cross-clamped in transgenic (group A1) and wild-type control mice (group B1) by the use of a permanent Yasargil-Aneurysm-Clip (FD 582; Aesculap-Werke AG, Tuttlingen, Germany). Complete clamping was verified under the operating microscope (× 20). Sham-operated control animals (group C1) underwent a similar procedure but did not undergo ischemia. An i.p. catheter (Abbocath-T, 20G × 32 mm; Abbott Ireland Ltd., Sligo, Ireland) was placed through the right abdominal wall and the laparotomy temporarily closed by disposable skin staples (Precise DS-25; 3M). Animals were kept under anesthesia on a heating pad (36.9 °C) in a left lateral position for 2 h. Skin staples were then removed and complete clamping as well as full reperfusion after removal of the clip was again checked under microscopic view (× 20). For the determination of endothelial integrity animals received 1.0 µCi ¹²⁵I-labelled bovine albumin (specific activity: 1 mCi/mg; ICN Pharmaceuticals Inc., Costa Mesa, Calif., USA) in 0.1 ml Ringer's lactate injected into the infrarenal vena cava. The laparotomy incision was closed again and reperfusion allowed for 3 h. After reopening of the laparotomy, terminal blood samples were taken from the vena cava to determine background radiation and organs harvested in the following manner. To expose the thoracic cavity, a bilateral longitudinal thoracotomy was performed. The inferior vena cava, the descending aorta, and the left atrial appendage were transected and the pulmonary artery intubated through an incision in the conus pulmonalis of the right ventricle. The pulmonary circulation was then flushed with 3 ml of Ringer's lactate by gravity infusion at 10 cm H₂O to omit background radiation. Lungs and heart were removed en bloc.

Permeability Index

Both lungs, a liver specimen of the median lobe, a gut specimen of the small intestine, both kidneys and quadriceps and triceps muscles from the right lower extremity were harvested, weighted, the radioactivity measured in the γ-counter (counts per minute (cpm); Cobra II, Auto-Gamma; Packard Instrument Company, Meriden, Conn.,

Fig. 1. Tukey box plot: Depicted are permeability index values of pulmonary tissue from animals after 2 h of lower torso ischemia and 3 h of reperfusion injury. The extent of the box indicates the 25th and 75th percentiles. The line inside the box shows the value of the 50th percentile. Capped bars indicate the 10th and 90th percentiles and symbols mark the 5th and 95th percentiles. Remote pulmonary ischemia-reperfusion injury is reduced in human C1 inhibitor overexpressing transgenic mice (group A1). No significant difference to sham-operated animals (group C1) is detectable, while wild-type control animals (group B1) show clear signs of endothelial disruption.

USA) and then dried to a constant weight at 85°C for 72 h. The permeability index (PI) is determined by the amount of extravasation of ^{125}I -labelled albumin and reflects a direct functional measurement of endothelial integrity. Permeability index values were calculated by the ratio (cpm/mg dry tissue)/(cpm/mg blood) as described [23, 24].

Liver Ischemia-Reperfusion

In a second experimental series, transgenic (group A2) and wild-type control mice (group B2) were subjected to liver ischemia-reperfusion, while sham-operated animals (group C2) did not undergo vascular clamping ($n = 4$ in each group). The abdomen was entered under general i.p. anesthesia and the entire hepatoduodenal ligament was cross-clamped for 30 min. Complete clamping as well as reperfusion after removal of the clip was then checked under microscopic view ($\times 20$) and 1.0 μCi ^{125}I -labelled bovine albumin injected intravenously. The laparotomy incision was temporarily closed and reperfusion allowed for 2 h. Tissue harvest and preparation was performed as described above. Permeability index values were then calculated.

Table 1. Permeability index values after lower torso ischemia (2 h) and reperfusion (3 h) injury

Organ	Group A1 (transgenic)	Group B1 (wild type)	Group C1 (sham)
Lung	0.53 ± 0.18	0.92 ± 0.29	0.42 ± 0.10
Skeletal muscle	0.08 ± 0.02	0.10 ± 0.02	0.06 ± 0.01
Liver	0.46 ± 0.11	0.51 ± 0.05	0.49 ± 0.12
Gut	0.06 ± 0.03	0.07 ± 0.03	0.06 ± 0.03
Kidney	0.97 ± 0.43	0.90 ± 0.07	1.07 ± 0.45

Mean of permeability index values (PI $\pm$ SD) after lower torso ischemia (2 h) and reperfusion (3 h) calculated from specific organ radioactivity (e.g. lung, skeletal muscle, liver, kidney, and gut). The permeability index allows a direct quantification of endothelial integrity.

Statistical Analysis

Results are expressed as mean $\pm$ SD. Data were analyzed statistically by Jandel Scientific Software (Jandel Scientific 1.0; San Rafael, Calif., USA). One-way analysis of variance was performed to compare groups and the Student-Newman-Keuls method applied for pairwise multiple comparison procedures. Kruskal-Wallis testing and pairwise comparison according to Dunn were used for non-normally distributed data. The significance level was set at $p < 0.05$.

Results

Lower Torso Ischemia-Reperfusion

Animals from all three experimental groups ($n = 18$) survived 5 h of narcosis.

In the skeletal muscle of the lower extremity, C1 inhibitor overexpression decreased the local endothelial cell damage significantly (group A1, PI = 0.08 ± 0.02) when compared to wild-type control animals (group B1, PI = 0.10 ± 0.02). No difference was seen between transgenic and sham-operated control mice (group C1, PI = 0.06 ± 0.01).

In the lungs of transgenic mice (group A1, PI = 0.53 ± 0.18), C1 inhibitor overexpression protected the vascular endothelium from remote ischemia-reperfusion injury as seen by a permeability index that was significantly lower than in wild-type control animals (group B1, PI = 0.92 ± 0.29) and not different to sham-operated controls (group C1, PI = 0.42 ± 0.10) (fig. 1).

In the liver, the gut, and the kidney, endothelial integrity after lower torso ischemia-reperfusion was unaltered and no difference in the permeability index between the three experimental groups was observed (table 1).

Table 2. Permeability index values after liver ischemia (30 min) and reperfusion (2 h) injury

Organ	Group A2 (transgenic)	Group B2 (wild type)	Group C2 (sham)
Lung	0.26 ± 0.01	0.28 ± 0.04	0.18 ± 0.04
Liver	0.46 ± 0.10	0.78 ± 0.07	0.50 ± 0.07
Gut	0.09 ± 0.02	0.15 ± 0.07	0.07 ± 0.01

Mean of permeability index values (PI ± SD) after liver ischemia for 30 min and reperfusion for 2 h.

Liver Ischemia-Reperfusion

In the series of hepatic ischemia-reperfusion all animals from the three experimental groups (n = 12) survived 2.5 h of narcosis.

We observed significant local hepatic endothelial cell protection against ischemia-reperfusion injury in transgenic C1 inhibitor overexpressing animals (group A2, PI = 0.46 ± 0.10) when compared to wild-type control animals (group B2, PI = 0.78 ± 0.07). No significant difference to sham-operated animals of group C2 (PI = 0.50 ± 0.07) was evident (fig. 2).

Clamping of the entire hepatoduodenal ligament caused portal blood flow obstruction. As a result significant elevation of the permeability index values in the gut of wild-type control animals (group B2, PI = 0.15 ± 0.07) were detected when compared to transgenic animals (group A2, PI = 0.09 ± 0.02) or sham-operated controls (group C2, PI = 0.07 ± 0.01) (table 2).

The permeability index values in the lungs after 30 min of liver ischemia and 2 h of reperfusion were statistically not different between the transgenic mice (group A2, PI = 0.26 ± 0.01) and the sham-operated animals (group C2, PI = 0.18 ± 0.04), whereas the permeability index values in the wild-type control mice was elevated (group B2, PI = 0.28 ± 0.04) indicating remote ischemia-reperfusion injury in this experimental group (table 2).

Discussion

Ischemia-reperfusion of the lower torso results in complement-mediated local and remote endothelial cell and organ injury [2]. The vascular permeability index allows a fast and accurate determination of organ-specific endothelial integrity [23, 24]. Local (i.e. skeletal muscle, liver) and remote (i.e. lung, kidney, gut) endothelial disruption

Fig. 2. Hepatic permeability index values in a Tukey box plot after 30 min of liver ischemia and 2 h of reperfusion injury. Ischemia-reperfusion injury is precluded by C1 inhibitor overexpression (group A2) as there is no significant difference to sham-operated animals (group C2).

can be assessed simultaneously in different models of ischemia-reperfusion injury. This approach helps to further elucidate organ-specific endothelial cell injury resulting from ischemia-reperfusion of local and distant organs.

We chose a severe lower torso ischemia-reperfusion injury model [21, 25]. While the mortality of 105 min of ischemia followed by reperfusion has been reported to be between 10 [23, 25] and 57% in mice [21], all animals of our series survived 120 min of ischemia and 180 min of reperfusion (n = 12). In contrast to the work of Nielsen et al. [21], we substituted fluid and electrolytes continuously (i.e. 0.2 ml Ringer's solution hourly for an adult mouse). During long-term narcosis in lower torso ischemia-reperfusion experimentation, fluid balance seems essential for small animal survival.

In a nonsurgical ischemia-reperfusion injury mouse model [15, 23] of 2 h of hindleg ischemia by external compression followed by 3 h of reperfusion after injection of ¹²⁵I-tagged albumin, permeability index values in sham control animals were comparable to sham-operated mice from our series. However, in the experimental group of

Kyriakides et al. [23] the application of external compression caused a massive endothelial disruption and permeability index values of 2.53 ± 0.11 , while in our series after infrarenal vascular occlusion a moderate but significant elevation of the vascular permeability was observed in wild-type animals (0.10 ± 0.02). Transabdominal induction of lower torso ischemia provides direct evidence of complete clamping and allows convincing visualization of reperfusion. Furthermore, the surgical approach excludes local muscular damage on the extremity examined.

Pulmonary endothelial cell disruption after radioactively labelled albumin injection is either measurable by comparing radioactivity in the aspirate after bronchoalveolar lavage within experimental groups [26, 27] or by determination of whole lung radioactivity after flushing of the pulmonary vascular bed as described in rats by Seekamp et al. [28]. From a pathophysiological point of view, extravasation into interstitial lung tissue is critical in the development of pulmonary edema. We therefore adapted the flushing procedure of the pulmonary artery and were able to determine pulmonary vascular leakage appropriately. In pilot experiments, flushing of the pulmonary artery with 10 cm H₂O gravity and 3 ml of Ringer's lactate was required to reduce background radiation. Intubation of the pulmonary artery under microscopic view proved to be easy and only one wild-type animal had to be excluded from further analysis due to technical difficulties during pulmonary artery perfusion.

Interestingly, Seekamp et al. [28] found in rats after 4 h of tourniquet-induced hindleg ischemia and 3 h of reperfusion considerably lower permeability index values in the lung (0.35 ± 0.05) than we determined in wild-type control mice (0.92 ± 0.29). We hypothesize that the two different ischemia-reperfusion injury models and the different species might explain the disparity of the results. Possibly rats are more resistant to remote pulmonary damage after lower torso ischemia-reperfusion injury than mice. Importantly, a pulmonary endothelial cell disruption was also found after 30 min of hepatic ischemia and 2 h of reperfusion. The endothelial damage was less pronounced than in the more severe lower torso ischemia-reperfusion model but indicates ongoing systemic complement-mediated remote organ injury in the hepatic ischemia-reperfusion model.

Mice overexpressing C1 inhibitor were protected in the lower torso ischemia-reperfusion locally in the skeletal muscle and remotely in the lungs against ischemia-reperfusion injury. We concluded that human C1 inhibitor overexpression caused complement modulation and en-

dothelial protection. As human C1 inhibitor is not only expressed in the serum of transgenic animals but also below the vascular endothelium [11], a local subendothelial complement inhibition might have enhanced the protection observed in transgenic animals.

In the hepatic ischemia-reperfusion model we also observed a local (hepatic and intestinal) and a remote pulmonary protection in transgenic animals. These results indicate that complement modulation by a transgenic approach can significantly reduce harmful sequelae of undesired complement activation during ischemia-reperfusion.

Transgenic animals might serve as tissue donors for allo- and xenotransplantation. C1 inhibitor overexpressing mice have already been used as recipients of rat cardiac xenografts, and it was shown that blockade of the classical complement cascade was transiently beneficial [29]. As the application of human C1 inhibitor was unproblematic in a variety of clinical disorders, one can speculate that a transgenic human C1 inhibitor overexpressing xenograft might also be tolerated well [3]. Furthermore, as coagulopathy seems to be another barrier to successful xenotransplantation [30], C1 inhibitor overexpression seems ideal due to its known capacity to inhibit the coagulation cascade and to prevent thrombosis [8]. With the technology of combining homologous recombination in fetal somatic cells with nuclear transfer [31], it may be worthwhile to induce overexpression of the human C1 inhibitor into a recombinant clonal pig donor organism. Control of the fluid-phase complement activation and inhibition of complement-mediated endothelial cell activation might contribute to long-term allo- and xenograft performance due to protection against inflammatory tissue injury.

In conclusion, we have developed surgical models of lower torso and liver ischemia-reperfusion injury in mice that allow simultaneous evaluation of local and remote tissue-specific endothelial disruption by the use of radioactively labelled albumin extravasation. Local and remote tissue injury was mitigated in transgenic human C1 inhibitor overexpressing mice in both series of ischemia-reperfusion indicating successful complement modulation by the transgenic approach.

Acknowledgement

We thank Dr. O. Stroka for critical revision of the manuscript.

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