Anti-CD163-dexamethasone protects against apoptosis after ischemia/reperfusion injuries in the rat liver

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HIGHLIGHTS

- We investigated the effect of pharmacologic preconditioning with HDD, LDD and anti-CD163-dex on ischemia/reperfusion injury.
- Liver cell apoptosis and necrosis were analyzed by stereological quantification.
- Anti-CD163-dex and high dose dexamethasone reduces the number of apoptotic cells following ischemia/reperfusion injury.

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ABSTRACT

Aim: The Pringle maneuver is a way to reduce blood loss during liver surgery. However, this may result in ischemia/reperfusion injury in the development of which Kupffer cells play a central role. Corticosteroids are known to have anti-inflammatory effects. Our aim was to investigate whether a conjugate of dexamethasone and antibody against the CD163 macrophage cell surface receptor could reduce ischemia/reperfusion injury in the rat liver.

Methods: Thirty-six male Wistar rats were used for the experiments. Animals were randomly divided into four groups of eight receiving anti-CD163-dexamethasone, high dose dexamethasone, low dose dexamethasone or placebo intravenously 18 h before laparotomy with subsequent 60 min of liver ischemia. After reperfusion for 24 h the animals had their liver removed. Bloods were drawn 30 min and 24 h post ischemia induction. Liver cell apoptosis and necrosis were analyzed by stereological quantification.

Results: After 24 h reperfusion, the fraction of cell in non-necrotic tissues exhibiting apoptotic profiles was significantly lower in the high dose dexamethasone (p = 0.03) and anti-CD163-dex (p = 0.03) groups compared with the low dose dexamethasone and placebo groups. There was no difference in necrotic cell volume between groups. After 30 min of reperfusion, levels of haptoglobin were significantly higher in the anti-CD163-dex group and high dose dexamethasone groups. Alanine aminotransferase and alkaline phosphatase were significantly higher in the high dose dexamethasone group compared to controls after 24 h reperfusion.

Abbreviations: Anti-CD163-dex, anti-CD163-dexamethasone; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate transaminase; BR, bilirubin; GGT, gamma-glutamyl transferase; HDD, high-dose dexamethasone; HE, hematoxylin & eosin; Hp, haptoglobin; IL-1, interleukin 1; IL-6, interleukin 6; IRI, ischemia/reperfusion injury; LDD, low-dose dexamethasone; MP, methylprednisolone; NVR, necrotic volume ratio; PM, pringles maneuver; ROS, reactive oxygen species; SURS, systematic, uniform, random sampling; TNF-α, tumor necrosis factor α.

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Conclusions: We show that pharmacological preconditioning with anti-CD163-dex and high dose dexamethasone reduces the number of apoptotic cells following ischemia/reperfusion injury.

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1. Introduction

The level of intraoperative blood loss and the transfusion requirement are factors known to be associated with the degree of morbidity and mortality following liver resection [1,2]. Moreover, these factors are important determinants of the morbidity, mortality and other postoperative complications and the risk of tumor recurrence [3–9]. Several surgical procedures have been used during liver resection in an attempt to reduce excessive perioperative bleeding [10]. The most common is Pringle’s maneuver, using inflow occlusion by portal triad clamping [11,12]. Lack of oxygen in the tissue leads to cell damage [13]. This can lead to important clinical problems including liver dysfunction and liver failure [14].

Ischemia/reperfusion injury (IRI) in the liver results from direct cellular damage caused by hypoxia and by the associated inflammatory tissue response, that results in further tissue damage and dysfunction [15,16]. The ischemic insult results in metabolic disturbances, caused by lack of oxygen, ATP-depletion and glycogen consumption [17].

An excessive inflammatory response during reperfusion plays an essential role determining the degree of IRI [14]. IRI occurs in two distinct phases after reperfusion, an early phase within the first 2 h and a later phase after 6–48 h [18]. In the early phase, reactive oxygen species (ROS) mainly produced and released from Kupffer cells aggravate the ischemic injuries [19,20]. In addition, an increased production of cytokines and chemokines can be detected. Signaling from ROS, cytokines and chemokines, recruits neutrophil granulocytes to the hepatic sinusoids, resulting in both local and systemic inflammation [21,22]. Late phase IRI is dominated by neutrophil infiltration, microcirculatory disturbance, and further damage to the liver [23–25].

Surgical intervention, pharmacological agents and gene therapy have been investigated as preventative strategies against IRI [26]. Pharmacological studies based on the pathophysiology of IRI have focused on the use of corticosteroids and antioxidants. Steroids induce an anti-inflammatory effect by providing negative feedback on immunological stimulation, stabilizing cell membrane integrity by inhibition of lysosome peroxidation and through regulation of gene expression pathways [27,28]. Corticosteroids seem to have a positive impact on the extent of IRI in the liver through an anti-apoptotic effect [29–33].

A possible pharmacological method to reduce IRI could be a newly developed antibody drug conjugate (ADC) targeting dexamethasone to the receptor CD163 expressed on the surface macrophages and monocytes, anti-CD163-dexamethasone [34–36]. The primary function of CD163 is as a scavenger receptor for the hemoglobin–haptoglobin (Hb–Hp) complex formed after the lysis of red blood cells [37]. Kupffer cells are liver-resident macrophages that contribute to activation of the immune system by releasing cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin 1 and 6 (IL-1 and IL-6) and by the production of ROS [38]. Targeting the CD163 receptor in macrophages highly increases the anti-inflammatory potency of dexamethasone. This has been tested in a rat and porcine model of inflammation after administration of lipopolysaccharides [34,36]. In these studies anti-CD163-dex at low dose was comparable to the anti-inflammatory effect of high dose free dexamethasone.

Given that Kupffer cell activation may result in inappropriate immune system up-regulation crucial to the pathogenesis of IRI, we hypothesized that anti-CD163-dex at a lower dose than free dexamethasone, might protect the liver from ischemic damage, thus preventing some of the well-known adverse effects of high dose glucocorticoids. We investigated this in the rat liver.

2. Methods

2.1. Experimental design

All experimental protocols used in this study were approved by the Danish Animal Experiments Inspectorate according to license number 2009/561-1644 and followed the rules of the “Guide for the Care and use of Laboratory Animals” published by the National Institutes of Health. In the experiment, we used 36 male Wistar rats (Taconic A/S, Bagsvaerd, Denmark) with an average weight of 225 g (range 195 g–301 g). The rats were housed in standard animal cages in an environmental- and temperature-controlled facility, at 23 °C and with a 12-h light–dark cycle. They were given free access to water and standard food (Altromin®).

Anesthesia was achieved using a mixture of oxygen (2.0 l/minute) and nitrous oxide (0.5 l/minute) containing 4.5% sevoflurane (forene; Abbott Laboratories, UK), blown through an induction chamber. During tail vein injection and surgery, anesthesia was maintained with 3% sevoflurane in the same mixture of oxygen and nitrous oxide, administrated through a mask covering the nose of the rat.

The animals were randomly divided into four groups receiving, respectively, anti-CD163-dex, high dose dexamethasone (HDD), low dose dexamethasone (LDD) or PBS intravenous through the tail vein 16–18 h before liver ischemia. During surgery, the rat was in a supine position on a temperature-controlled heated pad to maintain the core temperature at 37 °C. A transverse abdominal incision was made right under the rib cage. Under a dissecting microscope, all the hepatic ligaments were resected to achieve abruption of the collateral blood supply. In addition, the hepatoduodenal ligament was identified. A partial hepatic ischemia model was used to prevent mesenteric congestion by allowing blood flow through the right liver lobe [39].

Portal triad clamping was performed with a micro vascular clamp after the bifurcation of the right lobe, interrupting the portal triad flow to the left and median lobes. Discoloration of the lobes was used as a positive marker indicating hepatic ischemia. The abdomen was temporarily closed and the respiratory rate was measured every 10th minute to avoid major loss of fluid and bradypnea. As analgesics, the animals were given a subcutaneous injection of carprofen 5 mg/kg (Rimadyl®; Pfizer Animal Health, Exton, USA) and given intravenous isotonic saline 1 ml. After 30 min of ischemia, a blood sample was collected through the lateral tail vein to monitor the acid-base status and blood lactate concentration.

After 60 min of ischemia, the clamp was removed and the blood flow was re-established to the median and left lobes, verified by the return of the normal reddish color of the liver. The abdomen was closed in two layers, the fascia-layer with continuous suture and the skin-layer with single knots. After 30 min of reperfusion, a blood sample was collected. No antibiotics were administrated during the study period.
After 24 h of reperfusion the animals were re-anesthetized as described above. The abdomen and thoracic cavity were opened, and cardiac cannulation was performed to collect blood. Subsequently, the animals were euthanized by an intra cardiac injection of 1 ml pentobarbital 400 mg/ml. To ensure rapid fixation of the liver, the animal was perfused with phosphate-buffered 4% formaldehyde for 10 min through a cardiac cannula. The left portion of the median lobe was then resected and preserved in the same fixative for an additional 24 h, after which the lobe was embedded in paraffin.

2.2. ADC synthesis

Anti-CD163-dexamethasone (AD) was produced by conjugating dexamethasone hemisuccinate-NHS to primary amino groups of the rat CD163 antibody Ed-2 (ABD Serotec, Düsseldorf, Germany) as described [34].

Dexamethasone phosphate (Sigma–Aldrich, Broendby, Denmark) dissolved in PBS and PBS-buffer (Invitrogen, Taastrup, Denmark) was filtered and stored as AD, the study was conducted blinded [34].

2.3. Stereological sampling

The formaldehyde-fixed left median lobe was cut into 2 mm thick parallel slabs using a tissue slicer. All slabs were placed horizontally with the same side down in a cage before paraffin embedding allowing correct estimation [40]. A 2 μm section was cut from each paraffin embedded block, creating a set of systematic, uniform, random sampling (SURS) sections for analysis.

2.4. Immunohistochemistry

Apoptotic cells were identified by immunohistochemical staining for caspase 3. This cytosolic molecule is an effector protein, which causes DNA fragmentation and cell death [41]. Immunohistochemical staining was performed on 2.5 μm paraffin slides using a Benchmark XT automatic stainer (Ventana Medical Systems, Roche Group, AZ, USA). The primary antibody used in this study was Asp175 (clone5A1E, 1:80 dilution; Cell Signaling Technology, Glostrup, Denmark) against the activated form of caspase 3. Bound antibody was detected using the UltraView DAB detection kit (DAKO, Glostrup, Denmark) against the cardiac cannulation at 24 h of reperfusion were processed and embedded for an additional 24 h, after which the lobe was embedded in paraffin.

2.5. Stereological quantification of IRI

All sections were blinded to the investigator and analyzed using a microscope (Olympus BX-50) modified for stereology, with a motorized stage (Prior H138 with controller H29, Cambridge, UK) and a digital camera (Olympus camera DP72) connected to a PC with newCAST 3.6.5.0 software (Visiopharm, Hørsholm, Denmark.) The same investigator analyzed all sections. Intra-observer variance of stereological data was tested by re-evaluating multiple randomly chosen livers as described below.

2.6. Volume estimation

Volumes were estimated in hematoxylin and eosin (H&E)-stained SURS sections by the Cavalieri method [42]. A point grid was placed randomly over the H&E section using the software, and the areas of the entire section and of the necrotic tissue were estimated, as described [43]. To estimate the volume the following formula was used:

\[ V = t \cdot \frac{a}{p} \cdot \sum_{i=1}^{n} P_i \]  

(1)

\( t \) was the distance between each sampled section (2 mm = 2000 μm); \( (a/p) \) the area covered by each test point; \( \sum P_i \) was the number of points counted.

The necrotic volume ratio (NVR) was analyzed for each liver sample. NVR was calculated as:

\[ \text{NVR} = \frac{V_{\text{necrotic}}}{V_{\text{Total}}} \times 100\% \]

(2)

Results were adjusted for volume shrinkage as described [43].

2.7. Caspase 3 quantitation

Two-dimensional quantitation of caspase 3-positive cell profiles was performed on SURS sections from each animal as described [42]. Caspase 3-positive cell profiles were estimated by counting the number of positive cells within an unbiased counting frame. An average of 150 randomly selected, non-overlapping fields of view, were analyzed in each animal. The fraction of caspase 3-positive cell profiles (QA) was calculated using the following formula:

\[ QA_{\text{variable}} = \frac{Q}{A} = \frac{\sum Q(Casp3)}{a(mm^2) \cdot \sum P \cdot f_{\text{variable}}} \]

\[ f_{\text{variable}} = 1 - \frac{a_{\text{necrotic}}}{a_{\text{Total}}} \]

(3)

in which \( Q \) is the number of caspase 3-positive cell profiles; \( a \) is the area of the counting frame; and \( P \) is the number of counting frames evaluated; \( f_{\text{variable}} \) is the fraction of non-necrotic liver tissue used to correct the reference area. The counting rules used are described in Fig. 1b.

2.8. Liver parameters

Blood samples obtained after 30 min of reperfusion and from the cardiac cannulation at 24 h of reperfusion were processed and stored at –80 °C until analysis. Alanine aminotransferase (ALT), alkaline phosphatase (AP), bilirubin (BR), gamma-glutamyl transferase (GGT) and haptoglobin (HP) were measured using Roche Hitachi Cobas 6000 (Roche diagnostics, Mannheim, Germany).

2.9. Inflammatory mediators

Plasma IL-1, IL-6 and TNF were measured using a specific rat IL-1 (eBioscience San Diego, CA, USA), IL-6 (RayBiotech, Norcross, GA, USA), and TNF immunoassays (Invitrogen, Carlsbad, CA, USA). Rat acute phase protein-2-macroglobulin was measured using a specific ELISA kit (Immunology Consultants Laboratory, Portland, OR, USA). All of the assays used in this study have previously been validated for use in rats and were performed as described by manufacturer’s instructions.

2.10. Statistical analysis

Statistical analyses were performed using SPSS 19 (SPSS Inc., Chicago, Illinois, USA). All data are expressed as mean (CI). Data were tested for normal distribution and analyzed by ANOVA. For comparison of the two groups, normal distributed data were tested with Student’s t-test. Nonparametrical data were tested using Kruskal–Wallis test, followed by the Wilcoxon Mann–Whitney Rank sum test. P-values <0.05 were considered significant.

The reproducibility of the stereological data was tested by re-
evaluation of all sections from respectively 11 and 8 randomly
chosen livers from the two analyses. Data from the
first (A1) and the second evaluations (A2) were compared using Wilcoxon Man-
median values of the groups. Apoptosis was significantly lower in the HDD (p = 0.026) and the anti-CD163-dex (p = 0.047) groups compared with the control group. b) A typical
study section. Cell profiles were counted if they stained positive for caspase 3, if the nucleus of the cell was visible and if the cell profile was inside the counting, touched the green inclusion lines and did not touch the red exclusion lines. Cell profiles counted are marked with a black arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

Four animals were excluded because of insufficient clamping of the blood supply to the liver evaluated by ALT values of less than 3000 U/L. One animal was excluded due to portal vein thrombosis.

3.1. Stereology

3.1.1. Apoptosis

After 24 h of reperfusion, the fraction of apoptotic cell profiles in the non-necrotic tissue were 530 cell profiles/mm² (240–821) in the control group, 219 cell profiles/mm² (83–354) in the anti-CD163-dex group, 188 cell profiles/mm² (158–218) in the HDD group, and 235 cell profiles/mm² (129–362) in the LDD group. Apoptosis was significantly lower in the HDD (p = 0.03) and the anti-CD163-dex (p = 0.05) groups compared with the control group. The mean number of apoptotic cell profiles was lower in the LDD group (p = 0.06) compared with the control group, but the difference was not statistically significant (Fig. 1).

3.1.2. Necrotic volume and volume ratio (NVR)

After 24 h of reperfusion, the average Vnecrotic was 600 (505–827) in the control group, 498 (161–801) in the anti-CD163-dex group, 626 (438–832) in the HDD group, and 625 (282–1247) in the LDD group.

The average Vtotal was 970 (768–1205) in the control group, 991 (853–1146) in the anti-CD163-dex group, 1021 (886–1328) in the HDD group, and 1087 (814–1745) in the LDD group.

After 24 h of reperfusion, the average NVR could thus be calculated to 63% (55–71) in the control group, 51% (33–68) in the anti-CD163-dex and HDD groups, and 62% (52–71) in the LDD group. We found no significant differences between the groups (Fig. 2).

3.2. Biochemistry

3.2.1. Liver parameters

As illustrated in Table 1, the post ischemic rise of ALT was significantly higher in the HDD group compared with the control group after 24 h of reperfusion. However, when testing for multiple groups (ANOVA) there was no significant difference between groups. After 30 min of reperfusion, we found no difference in ALT between groups. After 24 h of reperfusion, the mean AP level was significantly higher in the HDD group compared with the control group, and the mean level of HP was significantly higher in the anti-CD163-dex and HDD groups (Table 1).
3.2.2. Inflammatory markers

As shown in Table 2, we found no difference in the post-ischemic rise in IL-1. The average level of α-2-macroglobulin was lower in the anti-CD163-dex group compared with the control group, but this was not significant (p = 0.063). After 30 min of reperfusion, levels of TNF-α and IL-6 were close to the detection limit in all groups (Table 2).

4. Discussion

We investigated the effect of pharmacologic conditioning with HDD, LDD and anti-CD163-dex on IRI. We show that conditioning with anti-CD163-dex and HDD significantly decreased the number of apoptotic cell profiles after 24 h of reperfusion, compared with controls, but was without effect in relation to the amount of necrosis. Our results suggest that both HDD and anti-CD163-dex might have the same protective effect on the biliary epithelium.

Table 2

<table>
<thead>
<tr>
<th>IL-1 [pg/mL] (30 min)</th>
<th>Control (n = 10)</th>
<th>Anti-CD163-dex (n = 9)</th>
<th>HDD (n = 8)</th>
<th>LDD (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>679</td>
<td>898</td>
<td>761</td>
<td>923</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>(252–1089)</td>
<td>(485–1310)</td>
<td>(939–1590)</td>
<td>(420–923)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>α-2-macroglobulin [mg/mL] (24 h)</th>
<th>Control (n = 10)</th>
<th>Anti-CD163-dex (n = 9)</th>
<th>HDD (n = 8)</th>
<th>LDD (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.17</td>
<td>0.81</td>
<td>1.25</td>
<td>1.05</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>(0.87–1.48)</td>
<td>(0.53–1.09)</td>
<td>(0.65–1.84)</td>
<td>(0.50–1.60)</td>
</tr>
</tbody>
</table>

* indicates a significant difference compared with the control group (P < 0.05).
found after 24 h. We did not investigate the amount of inflammation in these experiments and, therefore, cannot decide on this matter. IRI is known to initiate a strong inflammatory response, while in contrast corticosteroids have anti-inflammatory properties [27,28]. However, corticosteroids are also known to increase HP levels, which might explain this apparent paradox [49].

Kupffer cells produce cytokines, important mediators of the inflammatory response [21,50,51]. We measured cytokines after 30 min. IL-6 and TNF-α were close to the detection limit and there were no differences in IL-1 between groups. Other studies have shown a fall in TNF-α levels when preconditioning with prednisolone 2 h after reperfusion [31], and a decrease in IL-6 3 h after reperfusion when preconditioning with MP [33]. One possible explanation for not detecting higher levels of IL-6 and TNF-α in our analysis could be that samples were taken early in the inflammatory response, i.e. after 30 min.

The exact mechanism of corticoid action on hepatic IRI is unknown. Steroids in general induce an anti-inflammatory effect by providing negative feedback on immunological stimulation, stabilizing cell membrane integrity by inhibition of lysosome peroxidation and through regulation of gene expression pathways [27,28]. Figuero et al. were the first to suggest that corticosteroid had a protective effect on IRI in their study from 1975 [52]. They suggested that this protective mechanism might be mediated by cell membrane protection, either through stabilization of the lysosomal membrane or by the inhibition of circulating toxic substances excreted from the liver during ischemia [52]. In the kidneys, dexamethasone has been shown to up-regulate Bcl-xL, down-regulate ischemia-induced Bax, and inhibit activation of caspase-9 and caspase-3, thereby reducing both apoptosis and necrosis. Dexamethasone has also been shown to decrease the number of infiltrating neutrophils and lower ICAM-1 [53]. However, Wang et al. suggested that prednisolone appeared to reduce IRI in the liver by suppressing cytokine release [31] and this might be the mechanism by which anti-CD163-dex acts in the present study. Although we could not detect any major rise in cytokines in the present study at the time of blood sampling, it is likely that this may be one way by which corticosteroids protect against IRI in the liver.

Clinical studies have shown a beneficial effect of systemic corticosteroid conditioning before liver surgery [32]. Moreover, Kotsch et al. [54] showed that MP therapy improved outcome after deceased donor liver transplantation, reducing inflammation in the donor organ. In liver transplantation, the degree of apoptosis is an important marker of outcome [55,56]. An anti-apoptotic effect of treatment with anti-CD163-dex might also improve the outcome in liver transplantation, opening the way for further therapeutic opportunities. Thus, if the severity of IRI can be substantially reduced, it might be possible to split the donor liver between more recipients.

In our study, rats were treated with a single dose of anti-CD163-dex, resulting in a reduction in the number of apoptotic cells. It is possible, that administering several doses of the conjugate before liver ischemia could enhance this protective effect, as well as timing of treatment could perhaps be optimized. One could also speculate that anti-CD163-dex might be a potential treatment of autoimmune liver disorders including primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis and IgG4-related liver diseases. In this way one might avoid many of the complications seen after long-term high-dose corticosteroid therapy. These considerations need further research.

A major strength of our study was that we used stereological quantification in a blinded and randomized fashion. This is in marked contrast to previous studies that have used a variety of semi-quantitative methods to assess IRI. Moreover, an intra-observer analysis found no significant differences in the study results. In addition, our study was limited by lack of data concerning the inflammatory markers due to timing of blood sampling. However, with the experimental model used, it was not possible to obtain more than one adequate blood sample before euthanisation.

In conclusion, our study shows that pharmacological conditioning with anti-CD163-dex and HDD reduces the number of apoptotic cells following IRI compared with controls. We found no differences with regard to the degree of necrosis. Our findings do not allow us to decide whether or not the anti-CD163-dex effect on IRI was caused by inhibition of the inflammatory response.

Ethical approval

The study was approved by the Danish Animal Experiments Inspectorate according to license number 2009/561-1644 and followed the rules of the “Guide for the Care and use of Laboratory Animals” published by the National Institutes of Health.

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Author contribution

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Kasper Jarlhelm Andersen: Study design, data analysis, writing, approval.
Jens Randel Nyengaard: Study design, data analysis, writing, approval.
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Pia Svenndsen: Data collection, data analysis, writing, approval.
Holger Jon Møller: Study design, data analysis, writing, approval.
Søren Kragh Moestrup: Study design, writing, approval.
Jonas Heilskov Graversen: Study design, data analysis, writing, approval.
Frank Viborg Mortensen: Study design, data analysis, writing, approval.

Conflict of interest statement

Holger Jon Møller, Søren Kragh Moestrup and Jonas Heilskov Graversen own shares in Affinicon, which holds IP protecting the use of CD163 drug targeting.

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References


