Prolonged C1-inhibitor administration improves local healing of burn wounds and reduces myocardial inflammation in a rat burn wound model.

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ABSTRACT

Objective: In a previous study we found persistent presence of acute inflammation markers such as C-reactive protein (CRP) and complement factors locally in burn wounds. This persistence of acute inflammation may not only delay local burn wound healing but also have a systemic effect, for instance on the heart. Here, the effects of C1 esterase inhibitor (C1inh), an inhibitor of complement activation, on burn wound progression and the heart were analysed in rats.

Methods: Dorsal full thickness burn wounds (2 x 4 cm) were induced on female Wistar rats (n = 14). The rats were divided into two groups (n = 7): a control group (just burns) and a C1inh group. C1inh was administered daily intravenously for 14 days. The burn wound, healthy skin from hind leg (internal control) and the heart were then fixed in formalin. Tissues were analysed granulation tissue formation, reepithelialisation, amount and type of infiltrating inflammatory cells (granulocytes and macrophages) and inflammatory markers (complement factors C3 and C4).

Results: C1inh treatment significantly reduced the amount of granulation tissue and significantly increased reepithelialisation. C1inh also significantly reduced macrophage infiltration. Burns induced infiltration of macrophages into the ventricles of the heart and remarkably also into the atria of the heart. This effect could be counteracted by C1inh.

Conclusions: These data show that systemic treatment with C1inh acts at different levels resulting in improved healing locally in burn wounds and systemically reduced inflammation in the heart. Therefore, C1inh might be a possible therapeutic intervention for burn wound patients.
INTRODUCTION

Burn injury is still a significant problem world wide. It is estimated that mild burns occur with an annual rate of 600 / 100 000 inhabitants, whereas severe burns, which can result in fatal complications including shock, infection, electrolyte imbalance, respiratory failure and severe emotional and psychological distress because of long-term hospitalization, scarring and deformity, occur with a rate of 5 / 100 000 inhabitants1. Especially severe burns induce a massive inflammatory response, both systemic and locally in the burn wound. This inflammatory response not only affects the healing process of the burn wound, but also exerts effects systemically in different organs beyond the skin including the heart2-5. As part of the acute phase response the complement system has been shown to negatively affect the pathophysiology of the burn wound patient, both locally in the burn wound and systemically. The complement system is a large family of effector and regulatory proteins that are a prominent component of the innate immune system. The involvement of complement in burn wound pathophysiology is now well accepted. Complement is upregulated both locally in the burn wound as well as systemically in response to burn injury6-9. Locally increased complement levels were shown to relate to increased vascular permeability10, vascular thrombosis and hypertrophic scar formation11. In addition, the complement system may target viable cells near the site of injury through the formation of the membrane attack complex, resulting in reactive cell lysis and deepening of the wound4. In fact, inhibition of complement activation through either the natural complement inhibitor C1-esterase inhibitor (C1inh) or genetic C4 knockout did reduce capillary leakage, wound depth and scarring in burned pigs and mice12-14, underlining the pathogenic involvement of complement in burn wounds. On a systemic level, complement activation was shown to relate to the severity of burn trauma, clinical outcome6,15,16, postburn sepsis and organ failure5.

An important organ that was shown to be affected by the post-burn inflammatory response is the heart. Both in humans and in several animal models, burn injury resulted in reduced cardiac function17-23, and increased cardiac inflammation. In rats, burn trauma was shown to induce an increase in plasma and cardiomyocyte levels of the proinflammatory cytokines such as interleukin-1β, tumor necrosis factor-α and interleukin-6 and related loss of contractile function21,24,25. Also complement activation has been associated with burn-related cardiac dysfunction, as inhibition of complement through C5a blockade (for 1, 6, 12 and 24 hours after burn injury) or C1inh reduced cardiac dysfunction, as measured via monitoring hemodynamics during the experiment, in animal burn models13,26.

Therefore, local and systemic involvement of complement in burn wound pathophysiology is well established and inhibition of complement has been shown to be beneficial on both these levels. In burn trauma, local and systemic complement levels are persistently upregulated for months after trauma9,27, and burn-related cardiac aberrations, namely significantly increased
cardiac output, heart rate, cardiac index and rate pressure, were reported in humans months after trauma\textsuperscript{23}. Studies investigating the effects of complement inhibition in burn trauma so far only analyzed relatively short-term effects, up to a maximum of 4 days after injury\textsuperscript{12-14,26}. In this study, the effects of extended complement inhibition were analysed at a later time point in a rat burn wound model with continuous C1inh treatment for 14 days. The effects of this treatment were examined on burn wound pathophysiology and on inflammation of the heart.

**MATERIALS AND METHODS**

**In vivo rat burn wound model**

Twelve-week-old female Wistar rats (n = 14) were anesthetized using isoflurane. A dorsal full-thickness burn wound (4 x 2 cm) was created on a shaved part of the skin (just below the shoulders) using a copper stamp heated to 100ºC for 15 seconds. Before this, the rats received Temgesic (buprenorfine, 0.05 mg/kg, subcutaneous) as an analgesic. After induction of the burn wound, the wound was covered with nonadhesive bandage. Seven rats received purified human plasma-derived C1inh (100U/Kg, Sanquin, Amsterdam, The Netherlands) daily intravenously. The rats were restrained using a restrainer and subsequently C1inh was injected in the tail vein (alternating between left and right tail vein). The burn-only rats were not subjected to this treatment.

After 14 days, the entire wound was excised in addition to healthy skin from the hind leg, fixed in formalin, and embedded in paraffin. Furthermore, heart tissue was excised, fixed in formalin and prepared for (immuno)histochemical analysis. As a control, healthy hearts from age- and sex- matched rats were analysed (n = 5).

Animals were treated according to the national guidelines and with permission of the Animal Experimental Committee of the VU University of Amsterdam.

**Immunohistochemistry**

For histochemical analysis, 4-µm sections were dewaxed, rehydrated and stained with hematoxylin and eosin. For immunohistochemical analysis, 4-µm sections were dewaxed, rehydrated and incubated in methanol/H\textsubscript{2}O\textsubscript{2} (0.3%) for 30 minutes to block endogenous peroxidases. Next, antigen retrieval was performed; incubation with 0.1% pepsin (in 0.02 M HCl) for 30 minutes at 37ºC for slides to be stained for CD68, C3 and C4 or by heat inactivation in citrate buffer (pH 6.0) for slides to be stained for myeloperoxidase and ED2. Followed by incubation with either rabbit anti-human/rat myeloperoxidase (1:50, Abcam, Cambridge, UK), mouse anti-rat CD68 (1:400, Serotec, Kidlington, UK), mouse anti-human C3 (1:300, cross-reacts with rat, Santa Cruz, California, USA), rabbit anti-human C4 (1:300, cross-
reacts with rat, Santa Cruz, California USA) or mouse-anti-rat resident macrophage subtype ED2 (1:200, a kind gift from Prof C.D. Dijkstra, Amsterdam, The Netherlands) for 1 hour at room temperature. Sections were then incubated with Envision (undiluted, anti-mouse and rabbit, Dako Cytomation, Eindhoven, The Netherlands) for 30 minutes at room temperature. Staining was visualized using 3,3’-diaminobenzidine (DAB, 0.1 mg/ml, 0.02% H2O2). Sections were then counterstained with hematoxylin, dehydrated and covered. With each staining, a phosphate-buffered saline control and an isotype control were included for each antibody. All these controls yielded negative results (data not shown).

Morphometrical analyses
Every tissue sample was analysed for the amount of granulation tissue, as a percentage of the total surface area, and amount of newly formed epithelium using MIRAX SCAN (Carl Zeiss Micro Imaging GmbH, Gottingen, Germany). The infiltrate of inflammatory cells and complement positivity were digitally quantified as the percentage of the surface area of the tissue slide, using Slidebook software (Intelligent Imaging Innovations GmbH, Gottingen, Germany) (version 5.0.0.8).

Statistical analysis
Statistics were performed with the SPSS statistics program (windows version 11.5, SPSS Inc., Chicago). For each dependent variable (CD68, myeloperoxidase, C3, C4 and ED2) a repeated measure analysis of variance was performed. Also post-hoc Bonferroni tests were conducted. Distribution data were compared by χ² analysis. Values at the p ≤ 0.05 level were considered significant.

RESULTS
Effect of C1inh on burn wound histology
The induction of the burns resulted in loss of the epidermis and extensive damage of the dermis resulting in granulation tissue formation. In the muscle layer underneath, minor inflammation was seen (Figure 1, small arrows). Burns induced a significant thickening of the dermis (p<0.001) compared with control skin (mean ± SE, control skin: 766 ± 23 micrometer; 1390 ± 67 and 1270 ± 60 micrometer in burn and C1inh groups, respectively; Figure 2A), although the thickness of the dermis in the C1inh group was decreased, as compared to the burn group. Next, the amount of granulation tissue was determined as a percentage of the total dermis. The total amount of granulation tissue was significantly less in the C1inh group compared with the burn group (p<0.001; burn: 65 ± 2 %; C1inh: 50 ± 2 %; Figure 2B). As an indication of wound healing, the amount of newly formed epidermis, as a percentage of the
total cross section, was measured (Figure 2C). In the C1inh group significantly more new epidermis was formed compared with the burn group (p<0.05; burn: 38 ± 4 %; C1inh: 55 ± 4 %; Figure 2D). Thus, these histological data indicate improved wound healing in the C1inh-treated rats.

Figure 1: Burn wound morphology 14 days after infliction
Example of a hematoxylin and eosin staining of a full-thickness burn wound, the thickened dermis, and the formed granulation tissue with its cellular infiltrate are indicated. Also indicated is the subdermal muscle layer with minor cellular infiltrate (small arrows). Magnification x100.
C1-inhibitor shows positive effects in burned rats

**Figure 2: Histochemical analysis of burn wounds**

(A) Thickness of the dermis (µm) in healthy skin (n = 14), untreated burn rats (burn; n = 7) and C1inh-treated burned rats (C1inh; n = 7). (B) The amount of granulation tissue as the percentage of the total dermis in burn injury (n = 7) and C1inh-treated rats (n = 7). (C) Hematoxylin and eosin staining example of newly formed epidermis (arrow) in a burn wound of a C1inh-treated rat. Magnification, x200. (D) Amount of newly formed epidermis in the untreated burn group (n = 7) and the C1inh-treated group (n = 7).

**The effect of C1inh on inflammation in the burn wound**

The effect of C1inh treatment on local inflammation was also analysed. As parameters of local inflammation, infiltrating macrophages and neutrophilic granulocytes were quantified as well as complement deposition. The infiltrating cells were quantified as a percentage of the total surface area.

Burns induced a significant increase in the amount of infiltrated macrophages in the dermis (p<0.01) compared with control skin (control skin: 1.2 ± 0.2 %; burn: 7.7 ± 0.9 %; C1inh: 4.8 ± 0.7 %; Figure 3A). In the C1inh group, the amount of infiltrating macrophages was significantly lower than in the burn group (p<0.05). The subtype of macrophages was also determined. Macrophages of the ED2 subtype are considered to be resident, antiinflammatory and healing-inducing, whereas macrophages of the ED1 subtype are considered to be proinflammatory. In the burn group, the percentage of ED2 subtype macrophages was 21 ± 7 % and did not
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Differ significantly compared with control skin (31 ± 6 %; Figure 3B). In the C1inh (50 ± 8 %) group the percentage of ED2 subtype macrophages was significantly higher than in the burn group (p<0.01).

In burned skin of both groups the amount of infiltrating neutrophilic granulocytes was significantly higher compared to control skin (p<0.001; control skin: 0.3 ± 0.0 %; burn: 1.3 ± 0.1 %; C1inh: 2.0 ± 0.2 %), although the amounts were markedly lower than the amounts of infiltrating macrophages (Figure 3C). Remarkably, the amount of granulocytes in the C1inh group was significantly but limited higher compared to the burn group (burn: 1.3 ± 0.1 %; C1inh: 2.0 ± 0.2 %; p<0.001).

Figure 3: Analysis of the infiltrate of inflammatory cells in the burn wounds

(A) Amount of CD68-positive cells (macrophages) as a percentage of the total surface area of the dermis in C1inh-treated (n = 7), healthy skin (control, n = 14) and untreated burn rats (n = 7). (B) The percentage of ED2 subtype macrophages of the total amount of CD68 positive cells C1inh-treated rats (n = 7), healthy skin (control, n = 7) and burn injury (n = 7). (C) Amount of MPO-positive cells (neutrophils) as a percentage of the total surface area of the dermis in burn injury (n = 7), C1inh-treated rats (n = 7) and healthy skin (control, n = 14).
Depositions of complement factors C3 and C4 were also quantified as the percentage of the surface area of the dermis, using Slidebook software (Figure 4). In control skin, only a very low amount of C3 and C4 was found (C3: 0.2 ± 0.1 %; C4: 0.1 ± 0.04 %). In burned skin, the amounts of deposited C3 and C4 were significantly increased compared with control skin (p<0.001; C3: burn: 2.7 ± 0.5 %; C1inh: 1.7 ± 0.2 %; C4: burn: 3.0 ± 0.3 %; C1inh: 2.6 ± 0.3 %). The amounts of deposited C3 and C4 in the C1inh-treated group were lower than in the burn group, although these differences were minor and not significant. Thus, C1inh treatment resulted in decreased inflammation in burn wounds, especially related to the number of macrophages.

Figure 4: Quantification of complement factors C3 and C4
(A) Amount of C3 positivity as a percentage of the total surface area of the dermis in C1inh-treated rats (n = 7), healthy skin (control, n = 14) and untreated burn rats (n = 7).
(B) Amount of C4 positivity as a percentage of the total surface area of the dermis in C1inh-treated rats (n = 7), healthy skin (control, n = 14) and untreated burn rats (n = 7).

The effect of C1inh treatment on inflammation in the heart
It is known that burn trauma can lead to cardiac dysfunction and cardiac interleukin production. Systemic inflammation resulting from burn trauma may be a contributor to this failure. However, histological analysis, revealed no obvious morphological changes (data not shown). Independent of the cause of heart failure, infiltration of macrophages was seen in nonburn-related failing human myocardium. Therefore, the number of infiltrating CD68-positive macrophages in the heart was quantified in both the ventricles as well as in the atria. As a control, the hearts (n = 7) of healthy sex- and age-matched nonburned rats were used.

In the burn group the number of macrophages was significantly increased in both the ventricles (Figure 5A) as the atria (Figure 5B) compared with control hearts (p<0.01 and p<0.001 respectively)(ventricles; control hearts: 6 ± 2 macrophages/mm²; burn: 38 ± 5; atria: control hearts: 9 ± 2; burn: 42 ± 8). In the C1inh-treated group, the numbers of intramyocardial macrophages in both the ventricles and atria were significantly lower than in the burn group.
(p<0.001; Figures 5A, B) (ventricles: 7 ± 1 macrophages/mm²; atria: 13 ± 2) and percentages almost returned to the levels observed in healthy, nonburned control animals.

In addition, the percentage of the antiinflammatory ED2 macrophages of the total amount of macrophages in the heart was determined. In both the ventricles and the atria of control hearts virtually all intramyocardial macrophages were of the ED2 subtype (ventricles: 97 ± 9 %; atria: 94 ± 38 %; Figures 5C, D). In the burn group, the number of ED2 subtype macrophages in the heart decreased compared with the controls. In the ventricles, the percentage of ED2 macrophages was significantly lower (p<0.001; 10 ± 3 %) and in the atria the percentage was also lower, but not significantly (19 ± 4 %). In the C1inh group, the percentages of ED2 subtype macrophages in both the ventricles and atria were significantly higher than in the burn group (p<0.05; ventricles: 27 ± 8 %; atria: 89 ± 22 %). Thus, C1inh treatment facilitated the transition to the antiinflammatory subtype of macrophages.

![Figure 5: Analysis of the inflammation reaction in the ventricles and atrias of the heart](image)

(A) Amount of CD68-positive cells (macrophages) in both ventricles of the heart as a percentage of the surface area in C1inh-treated rats (n = 7) healthy hearts (control, n = 5), and untreated burn rats (n = 7). (B) Amount of CD68-positive cells (macrophages) in both atrias of the heart as a percentage of the surface area in C1inh-treated rats (n = 7) healthy hearts (control, n = 5), and untreated burn rats (n = 7). (C) Amount of ED2 subtype macrophages as a percentage of the total amount of CD68-positive cells in the ventricles of the heart in C1inh-treated rats (n = 7) healthy hearts (control, n = 5), and untreated burn rats (n = 7). (D) Amount of ED2 subtype macrophages as a percentage of the total amount of positive CD68 cells in the atrias of the heart in C1inh-treated rats (n = 7) healthy hearts (control, n = 5), and untreated burn rats (n = 7).
DISCUSSION

The involvement of local and systemic activation of complement in burn wound pathophysiology is well established. Short-term pharmacologic and genetic inhibition of complement in the first day(s) after thermal injury was shown to have positive effects locally on both burn wounds and systemically on the heart\textsuperscript{12-14}. However, in burn wound patients, this activation of complement was found to be particularly prolonged\textsuperscript{9,27}. We now show in rats that long-term treatment (up to 14 days) with C1inh resulted in a decrease in the amount of granulation tissue, a decrease in local inflammation in the skin, improved wound healing and systemically in decreased cardiac inflammation.

Daily C1inh treatment for 14 days decreased the amount of granulation tissue formed after injury, indicative of reduced inflammation to the skin. It is known that burn-induced inflammation is involved in additional damage in burn wounds after thermal injury. Complement was shown to play an important role in this burn wound progression. Indeed, genetic knockout of complement factor C4 dramatically reduced burn wound severity in mice 3 days after burn injury coinciding with reduced neutrophilic infiltrate\textsuperscript{14}. As complement activation can lead to the chemotraction of neutrophilic granulocytes, which have been shown to contribute to postburn damage\textsuperscript{27,32,38}, inhibition of complement may limit the infiltration of neutrophilic granulocytes and thereby prevent postburn damage. In our study, 14 days after burn injury, we found only a limited infiltration of neutrophilic granulocytes that was not reduced in the C1inh group. However, we cannot exclude the possibility that the reduction in postburn damage observed in C1inh-treated rats was in part due to effects of C1inh on neutrophilic granulocyte infiltration earlier after burn injury\textsuperscript{33}. In our model, a significant increase in infiltrating macrophages, however, was observed 14 days after burn injury. C1inh treatment then significantly reduced macrophage infiltration into the burn wound. This decrease may be a direct result from the reduction in skin damage in C1inh-treated animals. Conversely, the proportion of the infiltrating macrophages that was of the antiinflammatory ED2 subtype, was significantly increased in C1inh-treated animals, raising the possibility that C1inh through this effect on macrophage subtype contributes to reduced postburn damage. In general, complement can also directly, through the formation of the membrane attack complex, induce death in cells independent of inflammatory cells\textsuperscript{34}. Therefore, C1inh may prevent burn wound progression through inhibition of direct complement-induced death of cells in the skin bordering the burned tissue. Prolonged C1inh treatment also increased the formation of new epidermis, indicative of accelerated wound healing. Accelerated wound closure in burn wounds is important, because it reduces fluid loss and the chance of infection and sepsis\textsuperscript{24,5}.

Next to this, obstruction of blood flow to the burned skin, eg, through increased vascular permeability, resulting in edema, and hypercoagulability, can also contribute to postburn
In addition to complement inhibition, C1inh can reduce vascular permeability. Indeed, it was shown that C1inh did reduce capillary leakage 2 days post-burn in pigs. Therefore, this capacity of C1inh may also have contributed to reduced postburn damage 14 days after burns in our rat model. Severe burns also have significant cardiodepressive effects. Reduced heart function after burn injury most likely is a complex cumulative result of a variety of different causes; both physiological and inflammatory. Physiologically, the severe fluid loss after burns, through increased (micro)vascular leakage and edema, can lead to intravascular hypovolemia and hypovolumic cardiac shock. In addition, burn-induced systemic inflammation can contribute to cardiac dysfunction through interleukin production and complement, as was shown in different animal burn models. In fact, the extent of systemic inflammation and cardiac dysfunction, as measured via tumor necrosis factor-α, interleukin-1β, interleukin-6 and troponin levels, seems to be related directly to the amount of damage in the skin. Among factors that may contribute to burn-induced cardiac dysfunction is complement activation, as inhibition of complement factor C5a and C1inh both improved cardiac function up to 4 days post-burn in rats and pigs respectively. In addition, left ventricular infiltration of neutrophilic granulocytes was observed in the first day postburn in rats. However, in all these animal studies, the role of burn-induced cardiac inflammation and dysfunction was analyzed only in the first day(s) after injury, whereas in humans, cardiac function and cardiac stress were found to be aberrant up to months after injury. We now show a significant increase in macrophage infiltration into both the ventricles as well as the atria of the heart, 14 days post-burn, indicative of persistent cardiac inflammation. This infiltration of macrophages into the heart was significantly counteracted by C1inh, and C1inh facilitated the transition to the antiinflammatory subtype of macrophages. Whether C1inh directly interferes with cardiac macrophage infiltration, or whether this is an indirect effect, through complement inhibition, remains to be established.

The significance of this myocardial presence of macrophages remains unclear. It has to be noticed that in our study, we did not measure heart function after 14 days and can therefore not relate this myocardial macrophage infiltrate directly to dysfunction, although intramyocardial macrophages have been related to chronic heart failure. The presence of inflammatory cells in the heart could be the result of cardiomyocyte death. Indeed, increased blood levels of cardiac protein troponin I were detected both in burn patients and animals, suggesting a certain amount of cardiac injury. However, studies in humans and animals showing that burn-related cardiac dysfunction is reversible appear to contradict these findings. Alternatively, intramyocardial macrophages may excrete cardiac depressing cytokines. Indeed, pro-inflammatory cytokines such as interleukin-1β, tumor necrosis factor-α and interleukin-6 have been shown to be involved in burn-induced contractile dysfunction in rats.
In conclusion, long-term treatment with C1inh after thermal injury leads to improved wound healing and decreased cardiac inflammation, pointing to C1inh as a possible therapeutic intervention in burn wound patients to improve not only local wound healing but also systemic consequences of burn injury. Furthermore, we also show that burns can induce infiltration of macrophages into ventricles and atria of the heart.
REFERENCES

C1-inhibitor shows positive effects in burned rats