APT070 inhibits complement activation during in vitro cardiopulmonary bypass

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Abstract

Background: The proteins of the complement cascade play an important role in inflammation and the immune response. They have been shown to be activated during cardiopulmonary bypass (CPB), and may be responsible for the inflammatory response to CPB. We looked at the effect of APT070, an anti-complement agent, on human blood during in vitro CPB.

Materials and methods: Four hundred millilitres of blood was venesected from healthy human volunteers and heparinised. To the blood was added either APT070 to a concentration of 50 μg/ml (n = 5) or vehicle control (n = 4). The blood was entered into an in vitro CPB circuit and circulated for 90 min.

Results: Our results showed that after 90 min of in vitro bypass APT070 significantly inhibited the activation of compliment as demonstrated by C3a (p = 0.03) and sC5b-9 (p = 0.01) levels, and reduced neutrophil stimulation as measured by CD11b expression (p = 0.04 at 90 min).

Conclusion: APT070 significantly inhibits complement and neutrophil activation. This result may have considerable implications, especially if it can be shown to decrease the inflammatory sequelae of CPB.

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1. Introduction

Cardiopulmonary bypass (CPB) is an invaluable surgical tool that has made possible a wide range of operations that were previously considered technically unachievable [1]. Despite these benefits, CPB is associated with causing significant morbidity in 1–2% of patients [2], the majority of which is attributed to the inflammatory response elicited by the bypass machine [1]. In the context of CPB, the complement system plays an important role in the inflammatory cascade that is driven predominantly by the alternative pathway [3]. This is further augmented by the classical pathway following protamine administration to reverse the effects of heparin at the termination of CPB [4].

The complement cascade comprises of more than 30 plasma proteins, and plays an important part in inflammation and the immune response [5]. Activation can be by the alternative or classical pathways, with inhibition by various glycoproteins. One of these inhibitors is the human complement receptor type 1 (CR1), a membrane-bound glycoprotein presenting in several allotypes [6]. The A-allotype is the most commonly found, and has an extracellular portion that consists of 30 repeating protein sequences known as short consensus repeat (SCR) domains or sushi domains. It exerts its effect by inhibiting C3 and C5 convertases and accordingly the dependent C3b and C4b. So by inhibiting C3 activation, it abrogates both the classical and alternative pathways of complement activation.

A protein with complement inhibitory effects but less potent than CR1 was produced by expressing the first 3 SCR domains of the A-allotype of CR1 in Escherichia coli [7]. Enhanced membrane targeting by using the myristoyl electrostatic switch process resulted in a compound APT070 (Adprotech Ltd, Saffron Walden, UK), with more than 100 times the potency of SCR 1—3 alone [8].

The first in vivo use of APT070 was in a shock-like rat model [9] where it showed a dose-responsive protective effect. Pharmacological studies show that APT070 is cleared moderately rapidly from the plasma compartment with a terminal half-life of approximately 1 h [10]. Clearance is marginally slower than the unmodified SCR 1—3, and binding to plasma proteins is relatively weak and reversible. Phase 1 studies in healthy humans have shown that APT070 is well tolerated when given intravenously [11], subsequent to which studies have begun to assess its role in rheumatoid arthritis and renal transplantation.
In the United States alone more than 400,000 operations using CPB are performed annually [12], and globally this figure is considerably larger. One to two per cent of this overall figure represents a significant number of patients that suffer complications as a result of the inflammatory effects of CPB.

We aimed to quantify the complement inhibitory effects and distribution of APT070 within an in vitro CPB circuit primed with human blood.

2. Materials and methods

2.1. Venesection

After obtaining local regional ethical committee approval and volunteer consent, nine healthy human volunteers (four males) were venesected using a sterile technique. Four hundred millilitres of fresh blood was collected in a venesection bag (Baxter Healthcare, IL, USA) and heparinised (CP Pharmaceuticals, Wrexham, UK) to a concentration of 300 IU/kg that is used clinically during CPB. The blood was placed in a roller pump (Cobe, CO, USA). Sodium chloride 0.9% (Baxter Healthcare) was used to prime the circuit that was agitated. Oxygen (BOC Gases, Manchester, UK) was supplied at a rate of 800 ml/min. Maintained at 37°C, and run at a flow of 800 ml/min. Oxygen (BOC Gases, Manchester, UK) was supplied at a rate of 800 ml/min.

Samples were collected from the dedicated sample port of the membrane oxygenator. Blood for cytokine, complement and APT070 assays were centrifuged at 1500 x g for 15 min. The resulting supernatant was removed and frozen immediately at minus 70°C for future batch analysis. These samples were taken prior and after drug/control addition, and after 2, 15, 30, 45, 60 and 90 min circulation within the circuit. Samples for CD11b assay were collected after the addition of drug/control, and 45 and 90 min after addition to circuit.

2.2. Drug administration

The concentration of APT070 was derived from a phase 1 study in humans [11] in which healthy volunteers were safely injected with 100 mg of APT070, resulting in a concentration in plasma after administration of 50 μg/ml. In the treatment group (n = 5), APT070 carried in vehicle (PBS, Dulbecco’s A, 50 mg/ml D-mannitol, 0.1 M L-arginine) was injected into the venesection bag just prior to entering the circuit, to give a concentration of 50 μg/ml. The blood was gently agitated to ensure thorough distribution. In the control group, an equal volume of vehicle alone was added to the blood, which was then gently agitated.

2.3. CPB circuit and sample collection

The CPB circuit comprised of a paediatric membrane oxygenator (D901 Lilliput, Dideco, Mirandola, Italy), a heat exchanger (Medtronic, MN, USA), and a single non-occlusive roller pump (Cobe, CO, USA). Sodium chloride 0.9% (Baxter Healthcare) was used to prime the circuit that was maintained at 37°C, and run at a flow of 800 ml/min. Oxygen (BOC Gases, Manchester, UK) was supplied at a rate of 800 ml/min.

2.4. Inflammatory markers

The inflammatory markers chosen were C3a, soluble C5b-9, interleukin-8 (IL-8), tumour necrosis factor (TNF), and CD11b. The anaphylatoxins C3a and C5b-9 have been shown to be useful markers of impending organ failure and length of hospital stay after CPB [13]. The cytokines IL-8 and TNF are indicators of inflammation and accepted surrogate markers of neutrophil activation that have been shown to rise during CPB [2]. CD11b is a subunit of the adhesion molecule Mac-1 in polymorphonuclear cells, which is also up-regulated during inflammation and neutrophil activation [14].

2.5. Assays

C3a and soluble C5b-9 were measured using ELISA (Quidel Corp., CA, USA), as were IL-8 and TNF (R&D Systems Europe, Oxon, UK).

A sandwich ELISA was used to quantitatively detect APT070 within plasma samples. ELISA plates were coated with monoclonal antibody 3E10 (Smithkline Beecham, Harlow, UK) at 10 μg/ml. Serial dilutions of plasma were added to the plate for 1 h at room temperature. Detection of bound APT070 was achieved by addition of 50 μg/ml biotinylated anti-APT154 (rabbit polyclonal, Adprotech, Saffron Walden, UK). After washing anti-biotin HRP is added for 1 h at room temperature and subsequent detection is achieved by incubation with TMB substrate (Sigma-Aldrich, Dorset, UK). A known APT070 concentration (50 ng/ml) with subsequent threefold dilutions was used to derive a standard curve, which allowed for quantitative analysis of the plasma samples.

Flow cytometry was used to detect CD11b. Heparinised blood samples were collected from the bypass circuit and 50 μl was immediately transferred to glass tubes containing 10 μl CD11b-FITC antibody (Beckman Coulter, High Wycombe, UK). The sample was vortexed thoroughly followed by 15 min incubation at room temperature in the dark. Five hundred microlitres of Optilyse C (Beckman Coulter) was then added to the tube and vortexed immediately. The sample was then placed in a 37°C water bath for 10 min. Following this incubation 50 μl phosphate-buffered saline was added, the sample vortexed, and allowed to stand for 5 min at room temperature. The sample was then analysed by flow cytometer (Epics XL, Beckman Coulter) using a protocol specifically set up for CD11b-FITC analysis.

2.6. Statistical analysis

Using the Analyse-it software package (Analyse-it, Leeds, UK), a Kruskal–Wallis test was applied to actual values for inflammatory markers at each time point. A non-parametric test was chosen due to the small sample size and because the data could not be assumed to be normally distributed. A p-value of ≤0.05 was taken to be significant.

3. Results

The aim of this work was to observe the effects of APT070 on inflammatory markers during in vitro CPB. These results...
show that our model of in vitro CPB causes activation of the complement cascade in human blood, as demonstrated by progressive rises in C3a (Fig. 1) and soluble C5b-9 (Fig. 2) in the control experiments over 90 min of bypass. These results are important as they imply that this in vitro model simulates the effect of in vivo CPB on complement activation [3,4] and so is an appropriate model in which to observe the effects of complement inhibition.

Our results show that APT070 significantly abrogated this inflammatory response, to the extent of virtually inhibiting any complement activation. These results are shown clearly in Figs. 1 and 2, and are significantly different ($p < 0.05$) between the APT070 group and control group from early time points.

The assays on APT070 concentration (Fig. 3) showed that there was a drop in APT070 concentration when the blood was entered into the CPB circuit, despite correction for any haemodilutional effect. This change in concentration was significant ($p = 0.03$); however, no significant change in concentration occurred in the 90 min of bypass ($p = 0.8$), suggesting that after initial distribution within the circuit, APT070 remains available.

Neutrophil function as measured by CD11b was also inhibited in the APT070 group (Fig. 4); however, this difference only became significant at the 90 min time point ($p = 0.04$). Interestingly, cytokine production did not show any correlation to the other inflammatory markers measured in this study (Figs. 5 and 6), and there was no significant

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**Fig. 1.** Median actual values of C3a for control- and APT070-treated groups show a significant difference between the groups at 30, 60 and 90 min. Asterisk (*) indicates time points with statistically significant difference between groups ($p < 0.05$). The error bars represent a single standard deviation.

**Fig. 2.** Median actual values for sC5b-9. Median percentage change from baseline of sC5b-9 in control and treatment groups shows a significant difference from 30 min onwards. Asterisk (*) indicates time points with statistically significant difference between treatment and control groups ($p < 0.05$).

**Fig. 3.** APT070 levels in experiments. Change in APT070 concentration in blood from venesection bag (pre-CPB) and during bypass. There is a significant reduction in APT070 concentration after the blood enters the circuit ($p = 0.03$), but no significant variation subsequent to this ($p = 0.8$).

**Fig. 4.** Median actual CD11b values (Relative Fluorescence Units, RFU). Neutrophil activation as measured by CD11b showed the inhibitory effect of APT070 at 45 min, which becomes statistically significant at 90 min ($p = 0.04$). The error bars indicate a single standard deviation.

**Fig. 5.** Median actual TNF levels. No significant difference in TNF expression was seen between the APT070 and control groups at any of the time points.

**Fig. 6.** Median actual IL-8 levels. No significant difference in IL-8 levels is seen between the APT070 and control groups for any time point.
difference between the APT070 and control groups. This result is discussed further in the following section.

4. Discussion

Overcoming the inflammatory sequelae of CPB may have considerable clinical benefits for many thousands of individuals every year, and by reducing postoperative complications there may also be significant cost benefits for health service providers. The role of the complement cascade in this inflammatory process is well established, but so far large clinical studies have produced variable results. Pexilizumab, a monoclonal antibody to human C5, has not shown a clear reduction in mortality or myocardial infarction in patients undergoing CPB [15]. However, Pexilizumab does not inhibit the formation of C3a, a potent anaphylatoxin that precedes the activation of C5, and whose pro-inflammatory effects cannot be ignored. Further evidence for this is provided by a study of TP10, which like APT070 inhibits complement through CR1, and showed a significant reduction in mortality and myocardial infarction in high-risk male patients requiring CPB [16]. APT070, like TP10, mimics the activity of human CR1, thus it is ideally placed to inhibit both C3 and C5 activation, and so theoretically allows for more complete complement inhibition.

The aim of this study was to assess the complement inhibitory effects of APT070 in an in vitro CPB model that activated complement. These results show that our in vitro model of CPB stimulates complement and neutrophil activation, as has been shown to occur in previous studies of in vivo CPB [13]. This is an important result as it validates our model as one that simulates the inflammatory effects of in vivo CPB, and as an appropriate model in which to assess the anti-complement effects of APT070. In vitro bypass circuits have been used extensively to investigate platelet [17] and leukocyte changes [18], and complement activation [19]. However, all in vitro CPB models have important differences when compared to in vivo CPB that must be taken into account when interpreting results. In particular there is only a fixed cellular component that is able to participate in the inflammatory response during in vitro CPB, with no replenishment of neutrophils and platelets as would happen in vivo. Furthermore, the effects of ischemia—reperfusion organ injury with the associated pathophysiological and biochemical changes [20], and the role of the endothelium in the inflammatory process cannot be reproduced in vitro, but obviously plays a significant role in vivo. Nevertheless, in vitro CPB is a cost-effective, safe and reproducible model that is invaluable in preliminary investigations.

Our results show that APT070 used at a concentration of 50 μg/ml effectively abrogates C3a and soluble C5b-9 production in human blood circulating through an in vitro CPB circuit. For both of the complement fractions measured, the difference in activation from baseline became more obvious with time, a greater statistical significance being attached to the latter time points. This may be due to a bypass time-dependent increase in complement activation, so that the difference between the two groups becomes more pronounced with time. It may also be due to the relatively small number of experiments performed, and a greater number may have increased the power of the study to show statistically significant differences at earlier time points.

The effect on complement activation is mirrored by our results for CD11b. This would suggest that neutrophil activation is also reduced by APT070. Neutrophil activation during CPB can be provoked by a number of mediators of which C3a and sC5b-9 are two. Neutrophil adhesion to endothelial tissue, and subsequent transmigration, is an important step in tissue injury and CD11b is an important adhesion molecule that is expressed on the surface of neutrophils and facilitates this process. Hence, it is also a reliable marker of neutrophil activation [14]. Previous studies have shown that at the onset of CPB there is a drop in neutrophil numbers due to haemodilution and absorption onto the protein layer in the extracorporeal circuit [21] and a subsequent increase in neutrophil numbers is due to release of immature cells from the bone marrow, and mobilization of marginated cell [22]. This is obviously not possible to observe in an in vitro model. Activated complement has been shown to be an important factor in neutrophil stimulation by the deposition of C5b-9 on the neutrophil surface [23]. Thus, it is entirely consistent with previous work that CD11b and complement are inhibited in the APT070 group. However, the inhibitory effect on CD11b only becomes significant after 90 min (p = 0.04) of bypass, which once again may be due to our small sample size.

Analysis of APT070 concentration shows a significant drop (p = 0.02) within 2 min of being introduced into the circuit despite correction for haemodilution, after which there is insignificant variation over the 90-min bypass period (p = 0.8). This would suggest that APT070 is distributed rapidly around the circuit, perhaps coating the synthetic surfaces as effectively as it does cell membranes. However, despite this drop in drug concentration it remains biologically active, as is evident by complement and CD11b inhibition.

The results for C3a and sC5b-9 would lead us to expect a similar trend with cytokines. Interestingly this is not seen, but there would appear to be inconsistencies in previously published work. In some studies cytokine levels have been undetectable [24], but other investigators have reported a rise in cytokines at the onset of in vivo CPB or when the cross-clamp is removed [25]. It maybe that IL-8 and TNF are degraded at a rate that at least matches production, or that a larger sample size would show a significant difference between the groups. Further work is needed to clarify this.

This study is a preliminary investigation into the anti-complement activity of APT070 during CPB. Although our results are encouraging, these results must be taken in the context of a small study using an in vitro model. However, a larger in vivo study may yet realise the full potential of APT070 as an anti-complement agent during CPB that may have significant benefits for patients and health service providers alike.

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References


