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Inhibition of corneal inflammation following keratoplasty by birch leaf extract

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ABSTRACT

The objective of this study was to determine the effect of birch leaf (*Betula pendula*) extract (BPE) on corneal inflammation following keratoplasty in the rat model. T cells were stimulated *in vitro* in the presence of BPE. Proliferation, activation phenotype and the number of apoptotic/necrotic cells in cell culture were analyzed by flow cytometry. Corneal transplantation was performed between Fisher and Lewis rats. Recipient rats were either treated with cyclosporine A at a low dosage (Low-dose CsA = LDCsA) or received LDCsA in combination with BPE (2×1 ml/day). Clinical signs for corneal inflammation and rejection time points were determined. Infiltrating leukocytes were analyzed histologically. BPE specifically inhibited T cell proliferation *in vitro* by inducing apoptosis. The phenotype was not affected. *In vivo*, BPE significantly delayed the onset of corneal opacification ($p < 0.05$). The amount of infiltrating $CD45^+$ leukocytes and $CD4^+$ T cells ($p < 0.001$) was significantly reduced by BPE, whereas infiltration of $CD163^+$ macrophages was not significantly different between the two groups. BPE selectively induces apoptosis of activated T cells. Accordingly, BPE treatment significantly reduces infiltrating T cells and subsequent corneal opacification following keratoplasty. Our findings suggest BPE as a promising anti-inflammatory drug to treat corneal inflammation.

1. Introduction

Corneal clarity is required for optimal vision and can be severely affected by any form of corneal inflammation. This reduction is mediated in the long-term by infiltrating leukocytes and pathological blood vessel formation. Independently of the causes, any occurrence of corneal inflammation must be treated, especially if the central cornea is involved. Once a corneal scar is established, keratoplasty becomes necessary to restore corneal transparency required for optimal vision.

Although the immune privileged status of the eye facilitates excellent graft survival rates, immune responses against a corneal transplant remain the major cause of irreversible graft failure with subsequent opacification and reduced visual acuity (Niederkorn, 2007; Streilein, 2003). If no inflammation exists prior to transplantation, very good outcomes are achieved regarding clear graft survival (<10% rejection) (Price et al., 1993). However, any form of ocular surface inflammation (e.g. following infection, alkalic burn etc.), systemically occurring immunological disorders (e.g. atopic dermatitis) or a young recipient age present an increased risk of rejection (Reidy, 2001; Schwartzkopff et al., 2010a). Depending on the individual risk factors, long-term rejection occurs in up to 85% of cases (Coster and Williams, 2005). This is mainly due to pre-existing corneal blood- and lymph-vessels and an increased frequency of pre-sensitized leukocytes at the ocular surface (Cursiefen et al., 2003; Streilein, 2003). In these patients, focused immunosuppressive therapy is required.

Corticosteroids are the mainstay of any suppression following keratoplasty, in order to reduce subsequent inflammatory corneal reaction. However, even if applied topically, they are associated with complications such as ocular hypertension and cataract development. In cases where corticosteroids are not sufficient, systemic treatment with cyclosporine A (CsA) and/or mycophenolate mofetil (MMF) were shown to significantly improve graft survival (Birnbaum et al., 2005; Reinhard et al., 2001). Even though

Abbreviations: BPE, *Betula pendula* extract; CsA, cyclosporine A; LDCsA, low-dose CsA; MMF, mycophenolate mofetil; CFSE, carboxyfluorescein diacetate succinimidyl ester; MTX, methotrexate; PI, propidium iodide.

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both drug treatments are more specifically directed against the evolving lymphocyte response to the graft, they involve a risk of hepatorenal dysfunction and infections as well as increased incidence of tumor development. Therefore, it is of great clinical interest to develop treatment strategies that have a maximal and specific effect on the respective immune mechanism and which also avoid side effects that could reduce quality of life.

The immune mechanisms of corneal allograft rejection have been intensively investigated in animal models. It is recognized that $CD4^+$ T cells are a major factor in graft rejection (Streilein, 2003). Although other leukocyte populations, such as macrophages (Slegers et al., 2000), NK cells (Claerhout et al., 2004; Schwartzkopff et al., 2010b), $CD8^+$ T cells (Niederkorn et al., 2006) or antigen-presenting cells (Saban et al., 2010) were also shown to influence the rejection time course, only the depletion of $CD4^+$ T cells promoted allograft survival in the long-term (Ayliffe et al., 1992; Yamada et al., 1999). In addition to these cellular components, the intensity of lymph- as well as hem-angiogenesis was also shown to be closely related to graft rejection (Hos et al., 2008). As T lymphocytes have the greatest impact during the inflammatory response following keratoplasty, many immunosuppressive medications aim to act on these cells. Aside from CsA or MMF, alternatives such as FK506 and FK778 also showed positive results (Birnbaum et al., 2007; Sloper et al., 2001). Most of these medications are associated with side effects if given at an effective dosage. Once dosage is reduced below a certain level, both side effects and drug effect cease at the same time. In order to minimize adverse effects but maintain drug efficacy, efforts have been made to combine a reduced dosage of the drug with additional substances that have similar properties (Hackstein et al., 2007).

Extract of birch leaves was reported to have an anti-inflammatory function (Klinger et al., 1989). We observed a cytostatic effect of a commercially available birch leaf extract (*Betula pendula* extract: BPE) on human lymphocytes *in vitro* (Gründemann et al., 2011). Therefore, we hypothesized that a combination of BPE with sub-therapeutic CsA dosage would effectively suppress corneal inflammation following keratoplasty. In this series of experiments, the immunosuppressive capacity of BPE was analyzed on rat T cells *in vitro* and in combination with a low dosage of CsA during the inflammatory response following corneal transplantation *in vivo*.

2. Material and methods

2.1. Cells and animals

Inbred female Fisher ($Rt1^{lv}$) and Lewis ($Rt1^{l}$) rats (Charles River, Sulzfeld, Germany) were used as donors and recipients of corneal transplants. All animals were handled according to EU Directive 2010/63/EU. For *in vitro* studies, T cells were isolated from spleens; monocytic cells were obtained from cell line U937.

2.2. Groups

Lewis rats aged 8 weeks were divided into two groups: Group 1 ($n = 13$) received daily cyclosporine A (Novartis AG, Basel, Switzerland) at a low, sub-therapeutic dosage (LDCsA) (1 mg/kg body weight). Group 2 ($n = 8$) received LDCsA together with BPE (2×1 ml/day), respectively. Therapy was administered intraperitoneally for 14 days. The investigated aqueous *B. pendula* extract (BPE) is an injectable plant extract from the leaves of the birch *B. pendula* Roth, which is marketed by ABNOBA GmbH (Pforzheim, Germany) as an officially registered preparation according to § 38/39 of the German Drug Law (Betula Folium D3 ABNOBA, batch-no. 706 A41 and 910 A06 was used in the present study). For legal reasons, the extract is manufactured according to method no. 32 of the German Homeopathic Pharmacopoeia (GHP). Harvested birch leaves undergo maceration in a patented pressing machine under protective atmosphere to avoid oxidation. The resulting BPE contains 1.88 mg of fresh plant material, corresponding to an anhydrous mass of 0.63 mg/ml. The finished product is sterile filtered, filled in glass ampoules and released for sale if it meets all current European Pharmacopoeia (EP) specifications for solutions intended for injection. All specifications for parental medications were fulfilled according to the EP. Good Manufacturing Practice (GMP) and quality control, defined by the EP, are monitored by the German authorities (Federal Institute for Drugs and Medical Devices; BfArM); this includes proof of plant source identity and absence of contamination by heavy metals, pesticides, aflatoxins and microorganisms. Cell biological experiments were performed at our laboratory in Freiburg, Germany using ampoules from the sales stock. For each experiment, a fresh ampoule of *B. pendula* extract was used and concentrations were tested as indicated.

2.3. Corneal transplantation and anesthesia

Anesthesia was performed with a short inhalation of isoflurane (ABBOTT GmbH&Co.KG, Wiesbaden, Germany), deepened by a combination of ketamine (Essex, München, Germany), xylazine (Bayer, Leverkusen, Germany) and atropine (Braun, Melsungen, Germany) intraperitoneally. Orthotopic penetrating keratoplasties were performed as described previously (Birnbaum et al., 2007). In brief, Fisher donor buttons (2.5 mm) were obtained and the animals were sacrificed afterward. Recipients were anesthetized as described above. The central cornea was removed using a 2.0 mm trephine. The donor cornea was fixed with 8 interrupted sutures (11.0 Ethilon, Ethicon, Norderstedt, Germany). Finally, a blepharorrhaphy was applied for three days.

2.4. Clinical graft assessment

After removal of the blepharorrhaphy, the grafts were examined by two independent investigators for signs of opacity, vascularization, and edema according to an internationally accepted scoring method (Birnbaum et al., 2007; Schwartzkopff et al., 2010c) explained in Table 1. Rejection was defined as complete opacification (grade 4). The animals were continuously monitored during the assessment for signs of toxic side effects such as weight loss.

2.5. Histological analyses

Four rats per group were sacrificed for immunohistological evaluation on day 9. $CD45^+$ leukocytes, $CD4^+$ T cells and $CD163^+$

Table 1
Clinical graft assessment. Evaluation of opacification, edema and vascularization (Birnbaum et al., 2007; Schwartzkopff et al., 2010c).

Opacification	
0	No opacity
1	Slight opacity, details of iris clearly visible
2	Moderate opacity, some details of iris no longer visible
3	Strong opacity, pupil still recognizable
4	Total opacity, pupil no longer visible
Vascularization	
0	No vessels
1	Vessels on host not in the transplant
2	Vessels in the periphery of the transplant
3	Vessels reaching the center of the transplant
Edema	
0	No edema
1	Slight edema
2	Strong edema, margin of the transplant slightly elevated
3	Severe edema, margin of the transplant elevated