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Analysis of parathyroid graft rejection suggests alloantigen-specific production of nitric oxide by iNOS-positive intragraft macrophages☆

Anja Matuschek [a,1], Michael Ulbrich [a,1,2], Stephan Timm [b], Manuela Schneider [a], Christoph Thomas Germer [c], Karin Ulrichs [a], Christoph Otto [a,*]

[a] Experimental Surgery, Experimental Transplantation Immunology, Clinic of General, Visceral, Vascular, and Paediatric Surgery (Surgical Clinic I), University of Würzburg Hospital, Oberdürrbacher Str. 6, D-97080 Würzburg, Germany

[b] University Medical Center of the Johannes Gutenberg University Mainz, Clinic of General and Abdominal Surgery, Langenbeck St. 1, D-55131 Mainz, Germany

[c] Clinic of General, Visceral, Vascular, and Paediatric Surgery (Surgical Clinic I), University of Würzburg Hospital, Oberdürrbacher Str. 6, D-97080 Würzburg, Germany

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ABSTRACT

Background: During acute rejection of organ or tissue allografts T cells and macrophages are dominant infiltrating cells. CD4-positive T cells are important for the induction of allograft rejection and macrophages are important effector cells mediating cytotoxicity via production of nitric oxide (NO) by the inducible NO-synthase (iNOS). In the present study we analysed whether the destruction of primarily nonvascularised parathyroid allografts is also mediated by iNOS-positive macrophages.

Methods: Hypocalcaemic Lewis rats received parathyroid isografts (from Lewis donors) and allografts (from Wistar Furth donors), respectively, under the kidney capsule. Levels of serum calcium above 2 mmol/L correlated with normal parathyroid function and below 2 mmol/L with parathyroid rejection. Accelerated parathyroid allograft rejection was induced by immunisation of Lewis recipients with the allogeneic peptide P1.

Results: Determination of serum calcium levels is a useful parameter to control parathyroid graft function, and therefore to determine allograft rejection. Macrophages positive for both major histocompatibility complex (MHC) class II molecules and costimulatory molecules accumulated in iso- and allografts, but iNOS-positive macrophages were only detectable in allografts in the presence of activated CD4-positive T cells. These results confirm a cooperation between activated T cells and intragraft macrophages to induce macrophage iNOS expression. Recipients immunised with the allogeneic peptide P1 demonstrated accelerated rejection of allografts (mean ± SD: 9.2 ± 0.9 days) in contrast to nonimmunised animals (mean ± SD: 15.8 ± 1.8 days). Allografts of P1-immunised animals were infiltrated faster by activated CD4-positve T cells and, in addition, the infiltrates of iNOS-positive macrophages were stronger than those in allografts of nonimmunised animals.

Conclusions: Intragraft iNOS-positive macrophages seem to be able to produce cytotoxic NO involved in the killing of allogeneic cells during the alloimmune response against primarily nonvascularised parathyroid organ grafts. Infiltrates of iNOS-negative macrophages found in parathyroid isografts were caused by antigen-independent inflammation triggered by surgically induced injury. The absence of activated T cells in isografts and their presence in allografts underlines their importance in inducing macrophage iNOS expression.

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1. Introduction

Macrophages play a critical role as both regulator and effector cells in infection, tumour growth, and wound healing dependent on the activation stimuli [1]. In the field of transplantation, macrophage accumulation has long been recognised as a feature of allograft rejection [2–4].

Nitric oxide (NO) is a small molecule derived from the amino acid L-arginine catalysed either by constitutively expressed NO-synthases

Abbreviations: IFN, interferon; IL, interleukin; iNOS, inducible NO-synthase; LEW, Lewis (rat strain); NO, nitric oxide; WF, Wistar Furth (rat strain).

☆ Author's contributions: AM and MU designed the study, set up the experiments, collected the data, co-drafted the manuscript and provided images and figures; ST analysed and interpreted the results; MS performed the RT-PCR analyses and macrophage stimulation assays; CG participated in editorial support and research funding; KU revised the article for intellectual content; CO assisted in the study design, experimental concept, and data interpretation, co-drafted the manuscript, designed the study, analysed and interpreted the results. All authors read and approved the final manuscript.

* Corresponding author. Tel./fax: +49 931 201 31712.

E-mail addresses: matuschek_a@chirurgie.uni-wuerzburg.dee (A. Matuschek), mail@dr-ulbrich.de (M. Ulbrich), timm@ach.klinik.uni-mainz.de (S. Timm), schneider_m@chirurgie.uni-wuerzburg.de (M. Schneider), germer_c@klinik.uni-wuerzburg.de (C. Thomas Germer), ulrichs_k@chirurgie.uni-wuerzburg.de (K. Ulrichs), otto_c@chirurgie.uni-wuerzburg.de (C. Otto).

[1] Contributed equally to this study.

[2] Current address: Klinikum Garmisch-Partenkirchen GmbH, Department of Internal Medicine, Auenstr. 6, D-82467 Garmisch-Partenkirchen, Germany.

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(NOS) or by inducible NOS (iNOS or NOS2). The local NO concentration influences its effects: NO generated primarily by iNOS reached toxic micromolar amounts, whereas NO produced by constitutively expressed NOS (eNOS and nNOS) is within nanomolar range [5]. Large amounts of NO react with the highly reactive superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$), which is highly toxic compared to NO and O_2^- [6]. The expression of iNOS is transcriptionally upregulated by bacterial lipopolysaccharide and proinflammatory cytokines, such as interferons (IFN) and tumor necrosis factor (TNF), and can generate high NO levels locally [7].

Hibbs et al. identified NO as an effector molecule of activated iNOS-positive macrophages [8] and elevated levels of NO were observed during the rejection of organ or tissue allografts [9–13]. In contrast, the inhibition of NO production promotes graft acceptance [14,15]. The results of these studies suggest that intragraft iNOS-positive macrophages are responsible for the production of NO. In the present study we show that both isografts and allografts were infiltrated by macrophages but only allograft-infiltrating macrophages were iNOS-positive. In addition, we present evidence that iNOS-positive intragraft macrophages are involved in the destruction of primarily nonvascularised parathyroid allografts.

2. Methods

2.1. Animals and transplantation groups

Male animals of the inbred strain Wistar Furth (WF; haplotype: $RT1^u$) weighing 250–300 g served as donors of parathyroid glands and male animals of the inbred strain Lewis (LEW; haplotype: $RT1^l$) as recipients (Harlan Europe, The Netherlands). The study was reviewed and approved by the Animal Care Committee of the local government in accordance with the European and national guidelines for animal care (German Law for the Protection of Animals). Two transplantation groups were formed: isograft (LEW → LEW) and allograft (WF → LEW).

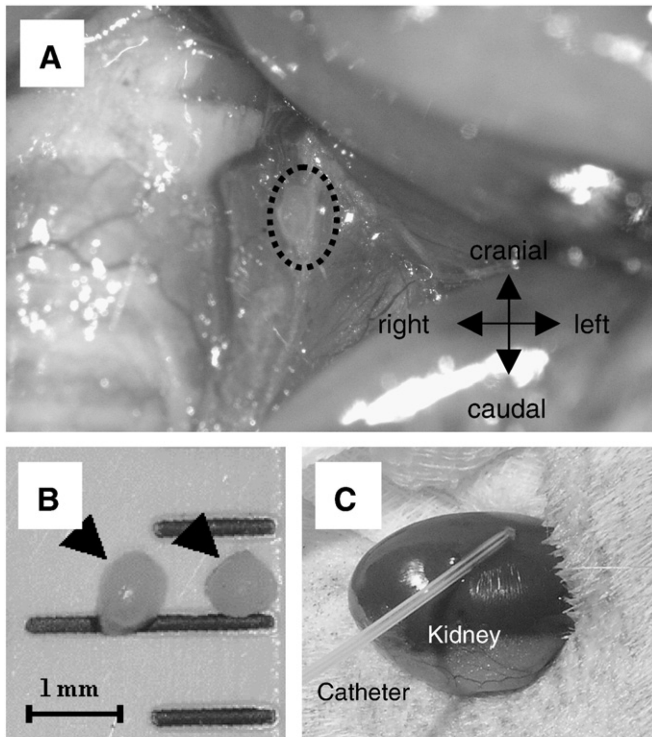

Fig. 1. The heterotopic parathyroid transplantation. Shown is the position of parathyroid glands in situ (A), their size (B), and the technique of heterotopic transplantation under the kidney capsule. The parathyroid glands were sited with a small catheter. For this, the left kidney was exposed by a lateral flank incision (C). Magnification in A and C, ×16.

Table 1
Primers and annealing conditions.

Primer		Sequence (5′ → 3′)	Size of PCR product (bp)	Annealing temperature (°C)
GAPDH[a]	Forward	GGT CGG TGT GAA CGG ATT TG	319	62
	Reverse	GTG AGC CCC AGC CTT CTC CAT		
iNOS[b]	Forward	GAT CAA TAA CCT GAA GCC CG	578	60
	Reverse	GCC CTT TTT TGC TCC ATA AGG		
CD40[c]	Forward	CGC TAT GGG GCT GCT TGT TGA CAG	401	58
	Reverse	GAC GGT ATC AGT GGT CTC AGT GGC		
CD80[d]	Forward	TGG TGA AAC ACC TGA CCA	517	50
	Reverse	GTT TCT CTG CTT GCC TCA		
CD86[d]	Forward	TGG GAA ACA GAG CTC TCA	518	53
	Reverse	AGG TTG ATC GAC TCG TCA		
PTH[e]	Forward	TAT GCG CTG GTG GAT GCG GA	539	52
	Reverse	AGG GAG GCG AGA GAC ATG GA		

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[b] Sterin-Borda L et al. *Neuropharmacology* 2003, 45:260–269.
[c] Matsui Y et al. *J Mol Cell Cardiol* 2002,34: 279–295.
[d] Giegerich R et al. *Proc Int Conf Intell Syst Mol Biol* 1996, 4:68–77.
[e] Tucci J et al. *J Mol Endocrinol* 1996, 17:149–157.

2.2. Isolation of parathyroid graft-infiltrating leukocytes

Four to six parathyroid allografts removed 7 days after transplantation from kidney capsule of transplanted LEW rats (see below) were washed two times with phosphate buffered saline (PBS; Gibco, Invitrogen), incubated with collagenase type IV (Sigma-Aldrich) for 30 min at 37 °C and subsequently meshed through a 70 µm cell strainer (Falcon, BD Biosciences Europe). The single-cell suspension was washed two times with PBS and subsequently centrifuged on slides for immunohistochemistry.

2.3. Isolation and stimulation of peritoneal macrophages

Peritoneal macrophages were collected from the peritoneal cavity. Euthanised LEW rats were injected intraperitoneally 10 mL ice-cold PBS [16]. The abdomen was massaged before PBS was collected. One million macrophages per well were seeded in a 24-well plate and after 2 h of macrophages adherence, nonadherent contaminating cells (red blood cells, lymphocytes) were washed out. The macrophages were stimulated with rat IFN-γ (1 µg/ mL; Miltenyi Biotech GmbH, Germany) overnight, and subsequently lysed with Trizol (Invitrogen Life Technologies GmbH, Germany) for RNA extraction.

2.4. Induction of alloreactive T cells

Some recipients of the allograft group (WF → LEW) were immunised with the allogeneic peptide P1 (P1 + allograft) 7 days before parathyroid transplantation. A mixture of 12.5 µl peptide (1 mg/mL) emulsified with equal volume of Titer Max® adjuvant (Enzo Life Sciences GmbH, Germany) was injected subcutaneously into each hind footpad. The synthetic allogeneic peptide P1 is a strong activator of alloreactive (anti-Wistar-Furth) LEW T cells and P1-immunised LEW rats reject accelerated WF cardiac allografts [17].

2.5. Parathyroidectomy and heterotopic parathyroid transplantation

The animals were anaesthetised with isofluran (induction, 3%; maintenance, 1.5%) (Abbott GmbH, Germany). The parathyroid glands located at the tip of the upper thyroid pole close to the upper thyroid pole vessels (Fig. 1A) were removed from LEW rats as described previously [18,19]. Parathyoidectomised LEW rats with stable hypocalcemia (below 1.9 mmol/L) for at least two weeks received 2

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