CHARLES UNIVERSITY IN PRAGUE

1ST FACULTY OF MEDICINE

THE ROLE OF T LYMPHOCYTES AND MACROPHAGES IN EXPERIMENTAL MODELS OF ALLO- AND XENOGRAFT REJECTION

(Summary of PhD thesis)

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- Holáň V, Krulová M, Zajícová A, **Pindjáková J**. Nitric oxide as a regulatory and effector molecule in the immune system. Molecular Immunology 2001; 38: 989-995.
- **Pindjáková J**, Vítová A, Krulová M, Zajícová A, Filipec M, Holáň V. Corneal rat-to-mouse xenotransplantation and the effects of anti-CD4 or anti-CD8 treatment on cytokine and nitric oxide production. Transplant International 2005; 18: 854-862.
- Holáň V, **Pindjáková J**, Zajícová A, Krulová M, Železná B, Matoušek P, Svoboda P. The activity of inducible nitric oxide synthase in rejected skin xenografts is selectively inhibited by a factor produced by grafted tissue. Xenotransplantation 2005; 12: 227-234.
- Holáň V, **Pindjáková J**, Krulová M, Neuwirth A, Frič J, Zajícová A. Production of nitric oxide during graft rejection is regulated by the Th1/Th2 balance, the arginase activity and L-arginine metabolism. Transplantation 2006; 81: 1708.
- Vítová A, **Pindjáková J**, Jirsová K, Zajícová A, van Rooijen N, Filipec M, Forrester JV, Holáň V. Macrophages and $CD4^+$ T cells are both required for acute corneal xenograft rejection. In preparation.

INTRODUCTION

Rejection of organs (tissues) by recipient's immune system remains a major obstacle in further development of clinical transplantations. In immunology research, as experimental models of transplantation, skin and cornea grafting are frequently used. Moreover, corneal transplantation, in striking contrast to the failure rate of the other grafts, became one of the most successful forms of tissue allotransplantation. The extraordinary success of corneal transplantation has been related to various features of the cornea and ocular microenvironment that together account for its immune-privilege status of anterior chamber.

The rejection of skin and corneal allo- and xenografts in untreated recipients is primarily studied as a model of acute rejection. This process begins after the first week of transplantation. Predominant cells infiltrating rejected grafts are both T cell subsets $CD4^+$ and $CD8^+$ (Illigens et al. 2002) and macrophages (Slegers et al. 2003, 2004, Axel et al. 2005).

It has also been described that corneal and skin allograft rejection is a $CD4^+$ Th1-cell-mediated process, demonstrated by increase level of IL-2 and IFN-γ (Hargrave at al. 2000) and low or absent of production and expression of genes for IL-4 and IL-10 (Satoru et al. 1998). After depletion of $CD4^+$ T cells, $CD8^+$ T cells can represent a source of cytokines required for graft rejection. IFN-γ, produced by both CD4+ and CD8+ T cells, is considered one of the most important cytokine of Th1-mediated rejection (Hidalgo and Halloran 2002).

The nature of cellular and humoral responses to xenogeneic tissues appears to differ from allograft immunity and is less understood. Majority of studies demonstrated that xenoreactive cellular response in acutely rejected xenograft is associated with production of Th1 and Th2 cytokines simultaneously (Dujovny et al. 2002, Tanaka et al. 2005).

Other studies brought evidence that macrophages are the key cytotoxic cell population in process of graft rejection (Yamamoto et al. 1998, Střeštíková et al. 2003), which are able to reject graft in the presence or absence of other effector cells (Slegers et al. 2003). Macropohages are multifunctional cells participate on both innate as well as adaptive immune response during. Macrophages activated by Th1 type cytokine IFN-γ produced nitric oxide (NO), an important effector and regulatory molecule in various models of immune response. NO is catalytically formed by several isoforms of NO synthases which converts L-arginine to NO and L-citrulin (Brüne et al. 1998). The role of NO as a cytotoxic effector molecule during allograft rejection has been well demonstrated when prolonged allograft survival was achieved by suppression of NO production by

selective iNOS inhibition (Holáň et al. 2001, Střeštíková et al. 2003) or NO scavenging (Roza et al. 2000).

On the contrary, macrophages stimulated by Th2 cytokines, the predominant cytokines of xenograft rejection, synthesize enzyme arginase (arginase I and/or arginase II). Both isoforms convert L-arginine into L-ornithine and urea. From this reason NO production may be reduced by arginase activity via depleting the common substrate in this cell types (Bronte et al. 2003). Thus, this regulatory mechanism of iNOS/arginase enzymes may regulate the effector mechanisms of transplantation reactions and can be important in xenograft rejection when the role of NO has not been well documented.

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AIMS

The main aim of this thesis was clarified the role of adaptive immune response, especially role of inflammatory cells infiltrating rejected graft - T cells and macrophages, their effector molecules, cytokines and enzymes (and their products) released during graft rejection.

Specific aims were:

- To investigate involvement and role of NO produced by macrophages in immune response to skin allografts in mice by using specific inhibitor of iNOS and demonstrate dependence of NO production on the presence of activated T cells.
- To show how treatment with mAb anti-CD4 or anti-CD8 affects the gene expression and production of Th1 and Th2 cytokines and iNOS in concordant corneal xenografts during acute rejection.
- To investigate the influence of local depletion of macrophages and/or T cells on rejection of corneal xenografts.
- To clarify the link between Th2 cytokine production and undetectable or only limited amount of NO in rejected skin xenograft.

SUMMARY OF RESULTS

NITRIC OXIDE AS A REGULATORY AND EFFECTOR MOLECULE IN THE IMMUNE SYSTEM

Holáň V, Krulová M, Zajícová A, **Pindjáková J**.

Molecular Immunology 2001; 38: 989–995

Nitric oxide (NO) as a small ubiquitous molecule influencing a great variety of biological processes in the organism. Within the immune system, increased levels of NO were observed in various immunopathological situations, inflammatory reactions and during the response to transplantation and tumor antigens. It appears that NO can influence various facets of immune response. We studied involvement and the role of NO in immune response to skin allograft in mice. The production of NO at the site of graft rejection correlated well with the kinetic of rejection reaction and with the fate of the allograft. Graft infiltrating macrophages were identified as a principal cell population producing NO and the production of NO by macrophages was dependent on the presence of activated CD4+ T cells. Survival of skin allografts was significantly prolonged by the treatment of graft recipients with 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), a specific inhibitor of inducible NO synthase (iNOS). These results suggest a role for NO as the effector cytotoxic molecule involved in the graft rejection. Experiments in vitro demonstrated that NO, in addition to its effector function, acts as a modulator of cytokine production. Spleen cells stimulated with alloantigens in the presence of AMT or *S*-ethylisothiourea (EIT), another selective iNOS inhibitor, produced considerably more interleukin (IL)-4 and IL-10 than the cells stimulated in the absence of iNOS inhibitors. The production of Th1 cytokines IL-2 and interferon (IFN)-γ was not enhanced by the inhibition of NO synthesis. The results altogether show that NO can act in transplantation reactions as an immunomodulator on cytokine production level and as an effector molecule involved in the graft destruction.

CORNEAL RAT-TO-MOUSE XENOTRANSPLANTATION AND THE EFFECTS OF ANTI-CD4 OR ANTI-CD8 TREATMENT ON CYTOKINE AND NITRIC OXIDE PRODUCTION

Pindjáková J, Vítová A, Krulová M, Zajícová A, Filipec M, Holáň V.

Transplant International 2005; 18:854-62

Corneal xenotransplantation may be an alternative approach to overcome shortage of allografts for clinical transplantation. Orthotopic corneal rat-to-mouse xenotransplantation and syngeneic transplantation was performed and the effects of anti-CD4 and anti-CD8 treatments on corneal xenograft survival and production of cytokines, interleukin (IL)-2, IL-4, IL-10, gamma-interferon (IFN-gamma) and nitric oxide (NO) were evaluated. RT-PCR was used to determine the expression of genes for cytokines and inducible nitric oxide synthase (iNOS) in the grafts. The presence of iNOS protein in grafts was detected by immunofluorescent staining. We found that corneal xenotransplantation was associated with a strong upregulation of genes for both Th1 and Th2 cytokines and with NO production in the graft. Treatment of xenograft recipients with mAb anti-CD4, but not anti-CD8, resulted in a profound inhibition of IL-2, IL-4 and IL-10 production, and in a significant prolongation of corneal xenograft survival. The results show that upregulation of Th2 cytokines after corneal xenotransplantation does not correlate with xenograft rejection. Rather, corneal graft rejection is associated with the expression of genes for IFN-gamma and iNOS and with NO production.

THE ACTIVITY OF INDUCIBLE NITRIC OXIDE SYNTHASE IN REJECTED SKIN XENOGRAFTS IS SELECTIVELY INHIBITED BY A FACTOR PRODUCED BY GRAFTED CELLS

Holáň V, **Pindjáková J**, Zajícová A, Krulová M, Železná B, Matoušek P, Svoboda P.

Xenotransplantation 2005: 12: 227–234

Abstract: Background: Production of nitric oxide (NO) by graft infiltrating macrophages has been suggested as an important effector mechanism of allograft rejection. Expression of the gene for the inducible NO synthase (iNOS) and the production of NO in rejected graft has been demonstrated in various models of allotransplantation. However, whether NO plays a role in rejection of skin xenografts has not been documented.

Methods: Explants of rejected skin allografts or xenografts (rat to mouse) were cultivated in vitro and the production of NO, interleukin (IL)-2, IL-4, IL-10 and interferon-γ (IFN-γ) by graft infiltrating cells was determined by the Griess reaction or ELISA. Effects of supernatants from cultures of xenograft explants on the expression of gene for iNOS, accumulation of iNOS protein and NO production were determined by RT-PCR or Western blots. Molecular mass of the factor with the suppressive activity was characterized by filtration on chromatography Sephacryl S-200 Superfine column. In addition, the effects of 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), a selective iNOS inhibitor, on survival of skin xenografts were tested.

Results: While explants of rejected mouse skin allografts produced substantial amounts of NO, undetectable or only very low levels of NO were found in supernatants from cultured rat skin xenografts. Cocultivation of bacterial lipopolysaccharide (LPS)-stimulated mouse macrophages which produce high quantities of NO, with pieces of rejected xenografts, but not of syngeneic grafts, allografts or normal rat skin, completely inhibited production of NO. Production of IL-6 and IL-10 by LPS-stimulated macrophages was not inhibited under the same conditions. The inhibition of NO production was mediated by a factor which was produced by rejected rat xenograft and which was eluted from chromatography Sephacryl S-200 Superfine column in a fraction representing a molecular mass of 67 kDa. The factor did not inhibit the expression of the gene for iNOS, reduce the level of iNOS protein in stimulated macrophages, or function as a scavenger of NO. Rather, the factor inhibited the function of iNOS. The finding that NO does not play an important role during rejection of skin xenografts is supported by the observation that treatment of graft recipients with

AMT, a specific iNOS inhibitor, did not enhance xenograft survival, while the same treatment resulted in prolongation of survival of skin allografts.
Conclusion: The results thus demonstrate that a 67-kDa molecule produced by rejected rat skin xenografts selectively inhibits iNOS activity in graft infiltrating macrophages. We suggest that NO does not play a significant role in rejection of skin xenografts as it does in the case of allograft rejection.

PRODUCTION OF NITRIC OXIDE DURING GRAFT REJECTION IS REGULATED BY THE TH1/TH2 BALANCE, THE ARGINASE ACTIVITY, AND L-ARGININE METABOLISM

Holáň V, **Pindjáková J**, Krulová M, Neuwirth A, Frič J, Zajícová A.

Transplantation 2006; 81:1708-1715

Background. Production of nitric oxide (NO) by graft infiltrating macrophages has been proposed as an important effector mechanism of allograft rejection. Although high levels of NO are generated during allograft rejection, undetectable or only limited amounts of NO were found in rejected skin xenografts.

Methods. BALB/c mice were grafted with skin transplants from syngeneic, allogeneic or xenogeneic (rat) donors. The production of NO, cytokines and arginase in the grafts was determined by spectrophotometry, enzyme-linked immunosorbent assay, or polymerase chain reaction. Effects of depletion of CD4+ cells, neutralization of interleukin (IL)-4 or application of arginase inhibitors N[omega]-hydroxy-L-arginine (L-NOHA) and L-valine on production of NO in rejected xenografts were evaluated.

Results. Rejection of rat skin xenografts, on the contrary to rejection of allografts, was associated with a local high production of Th2 cytokines IL-4 and IL-10, overexpression of arginase genes, strongly enhanced arginase activity and attenuated NO generation in the graft. The supernatants obtained after cultivation of skin xenograft (but not allograft or syngeneic graft) explants contained a high arginase activity and strongly suppressed NO production by activated macrophages. This suppression was completely inhibited by L-NOHA or was overcome by an excess of exogenous L-arginine, a substrate for NO synthesis. Cocultivation of xenograft explants that did not produce NO with arginase inhibitors L-NOHA or L-valine restored NO generation in the graft.

Conclusion. The results suggest that upregulation of arginase activity by Th2 cytokines during xenograft rejection limits the bioavailability of L-arginine for the inducible NO synthase and thus attenuates generation of NO by the graft-infiltrating macrophages.

MACROPHAGES AND CD4$^+$ T CELLS ARE BOTH REQUIRED FOR ACUTE CORNEAL XENOGRAFT REJECTION

Vítová A, **Pindjáková J**, Jirsová K, Zajícová A, van Rooijen N, Filipec M, Forrester JV, Holan V

In preparation

Background. To investigate the role of macrophages and T cells in rejection of corneal xenografts.

Methods. Corneas of rat Lewis strain were grafted orthotopically into mouse BALB/c recipients, which were, treated subconjunctivally with liposomes containing dichloromethylene diphosphonate (clodronate-LIP) alone or in combination with intraperitoneally administered monoclonal antibodies (mAb) anti-CD4 or anti-CD8. Expression of genes for mouse IL-2, IFN-γ, IL-4 and IL-10, rat endothelial nitric oxide synthase (eNOS) and mouse inducible NOS (iNOS) was determined in rejected corneal xenografts by RT-PCR. Organ-cultured graft explants were evaluated for the production of cytokines by ELISA and for the production of NO by the Griess reaction.

Results. Treatment of corneal xenograft recipients either with mAb anti-CD4 or clodronate-LIP significantly prolonged graft survival. However, combined treatment with anti-CD4 and clodronate-LIP was neither synergistic nor additive in its effect on graft survival. Depletion of CD8$^+$ cells had no effect on xenograft survival. Expression of genes for IL-2, IL-4 and IL-10 was reduced in corneal xenografts from recipients treated with mAb anti-CD4. In all recipients treated with clodronate-LIP inhibition of the expression of the gene for iNOS was observed. A significant expression of the gene for IFN-γ and production of cytokine IFN-γ was preserved in xenografts of rejecting recipients.

Conclusions. CD4$^+$ T cells and macrophages play an essential role in acute corneal xenograft rejection and their cellular reactions may be interdependent, suggesting that innate immune responses are not sufficient for a full xenograft rejection response. Rejection of corneal xenograft is associated with the production of IFN-γ.

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LECTURES

Pindjáková J, Krulová M, Holáň V. Stanovenie bunkových populácií a efektorových molekúl v kožných transplantátoch. 18. pracovní imunologická konference, Velké Karlovice, 19.-21. října 2001.

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