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Transcription and translation of inducible nitric oxide synthase in the pancreas of prediabetic BB rats

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The inducible NO synthase (iNOS) was found to be expressed in pancreatic lesions of adult diabetes-prone BB rats Pancreatic iNOS mRNA was detected by reverse transcriptase PCR in pancreatic RNA of adult diabetes-prone BB rats but not in normal Wistar rats, young diabetes-prone BB rats without insulitis or in diabetes-resistant BB rats. Immunohistochemistry of pancreatic sections using an iNOS-specific antiserum labeled the pancreas of adult diabetes-prone BB rats but not Wistar rats. Parallel staining for ED1-positive macrophages showed restriction of iNOS expression to areas of islet infiltration by macrophages. In conclusion, the data provide direct evidence for enhanced expression of inducible NO synthase in tissue lesions during the development of autoimmune diabetes.

Diabetes type 1; BB rat; Insulitis, NO synthase; Nitric oxide

1. INTRODUCTION

Nitric oxide (NO) has been identified in experimental models in vitro as a highly toxic compound for pancreatic islet cells. The lysis of islet cells by activated macrophages in culture is dependent on NO generated by the inducible NO synthase (iNOS) of macrophages [1]. The lytic activity of IL-1 towards isolated islets also requires iNOS activity [2]. Under non-lytic conditions IL-1 modulates insulin secretion after glucose stimulus via the induction of NO formation [3,4]. Finally, chemically generated NO was found to rapidly lyse islet cells, but was of lesser toxicity towards other cell types [5].

Although these and other findings have led to the suggestion that NO is a pathogenic factor in inflammatory or autoimmune diseases, direct evidence for enhanced iNOS expression at the site of local lesions is lacking for spontaneous inflammatory/autoimmune disease. We therefore studied diabetes-prone BB rats, which represent a spontaneous model of human type I diabetes. The disease in BB rats is macrophage- and T-cell-dependent. Insulin-dependent diabetes results from preferential β cell destruction during chronic progressive insulitis [6]. We demonstrate here the presence of iNOS in pancreatic lesions of prediabetic BB rats.

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Abbreviations: iNOS, inducible NO synthase; cNOS, constitutive NOS.

2. MATERIALS AND METHODS

2.1. *Animals*

Diabetes-prone and diabetes-resistant BB rats were obtained from Möllegaard Ltd., Eiby, Denmark (BB/Wor/Mol) and from the University of Massachusetts at Worcester, USA (BB/Wor/dp and BB/Wor/dr) at 4–5 weeks of age and maintained in our animal facility under standard conditions. Animals received tap water and rat chow (ssniff R, Ssniff, Soest, Germany) ad libitum. Breeding stocks of Wistar rats came from the Zentralinstitut für Versuchtierkunde, Hannover, Germany Diabetes-prone BB rats of both sexes were killed under anesthesia for pancreatic analysis at 5 weeks of age or between 9 and 11 weeks of age (when about 20% of littermates had developed diabetes). Diabetes-resistant BB rats and Wistar rats were killed between 9 and 12 weeks of age.

2.2. *mRNA analysis*

Vascular endothelial cells were isolated by outgrowth for 4–6 days from aortic rings on collagen gel following standard procedures [7]. Cells were subcultured for 3–6 passages, 10^6 cells were cultured on coverslips with or without 200 U/ml of human recombinant interleukin 1β (Genzyme, Cambridge, MA, USA) plus 100 U/ml rat interferon γ (HBT, Leiden, Netherlands) for 6 h. RNA was isolated from endothelial cells or from fresh pancreatic tissue by acid guanidinium thiocyanate–phenol–chloroform extraction [8]. Northern blotting of 10 μg RNA per lane was performed following standard procedures [9] using a cDNA of RAW 264.7 macrophage line iNOS [10] or a cDNA of endothelial cNOS [11] (kind gift of Dr. K.D. Bloch, Massachusetts General Hospital, Boston, USA). Of the iNOS cDNA the *Mbo*I–*Bam*HI fragment, bases 494–845, was used as probe, of the cNOS cDNA bases 1–606. Reverse transcriptase PCR was applied to detect iNOS mRNA in pancreatic RNA. Bases 200–866 were converted into cDNA using a specific primer, followed by polymerase chain reaction with 6 μg pancreatic RNA [12]. After a total of 35 cycles the product was subjected to electrophoresis on a 2% agarose gel followed by hybridization with iNOS and cNOS probes as described above.

2.3. *Immunohistochemistry and Western blot analysis*

Serial cryostat sections (5 μm) of pancreata were prepared, dried

and fixed with acetone at room temperature for 10 min Indirect immunohistochemistry was performed as described previously [13] The following antibodies were used: monoclonal mouse antibody ED1 (Serotec, Oxford, UK), rabbit hyperimmune serum to mouse macrophage iNOS [10], and peroxidase-based detection kits (Vectastain, Vector, Burlingame, USA) Slides were evaluated by two independent observers

Cultures of endothelial cells (see section 2.2) were dried and subsequently processed as described above For Western blot analysis approx 10^6 endothelial cells were lysed by 3% SDS buffer followed by electrophoresis on a 10% polyacrylamide gel, blotting and immunocytochemistry using standard protocols [14]

3. RESULTS

The cDNA probes of iNOS and cNOS were chosen from the N-terminal region since it contains the largest sequence differences between the two enzymes. As shown in Fig. 1A the iNOS and cNOS specifically detect inducible versus constitutive NOS message in endothelial cells. Using amplification by reverse transcriptase PCR the iNOS message was not detectable in non-activated endothelial cells, nor in pancreas of normal Wistar or diabetes resistant BB rats (Fig. 1B). In contrast, strong signals of iNOS mRNA were found in pancreata of diabetes-prone BB rats at 9–11 weeks of age, an age where all diabetes-prone BB rats exhibited insulitis (see immunohistochemistry below). Diabetes-prone BB rats did not express iNOS mRNA in the pancreas prior to

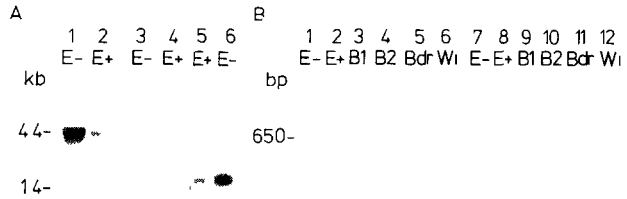

Fig. 1. (A) Demonstration of iNOS and cNOS mRNA in endothelial cells by Northern blot Lane 1+2, hybridization with cNOS probe; lane 3+4, iNOS probe, lane 5+6, β-actin probe. (B) Analysis for iNOS mRNA in pancreatic RNA by reverse transcriptase PCR. Lanes 1-6, hybridization of PCR products with iNOS probe; lanes 7–12, cNOS probe. E−, non-activated endothelium; E+, activated endothelium, B1, 5-week-old BB rat pancreas, B2, adult diabetes-prone BB rat pancreas; B dr, diabetes-resistant BB rat pancreas; Wi, Wistar rat pancreas In each case RNA of 3 different pancreata was analysed with identical results.

the onset of insulitis, at an age of 5 weeks (Fig. 1B). The absence of insulitis was verified by histological analysis.

Translation of iNOS transcripts in adult diabetes-prone BB rats was analysed by an iNOS-specific antiserum. Control experiments with resident and activated endothelials confirmed specificity of the antibody (Fig. 2). Staining for iNOS was found in pancreatic sections of adult diabetes-prone BB but not in control Wistar rats. In order to localize iNOS expression two serial pancreas sections were stained in parallel for macrophage infiltration (ED1 marker) and iNOS expression.

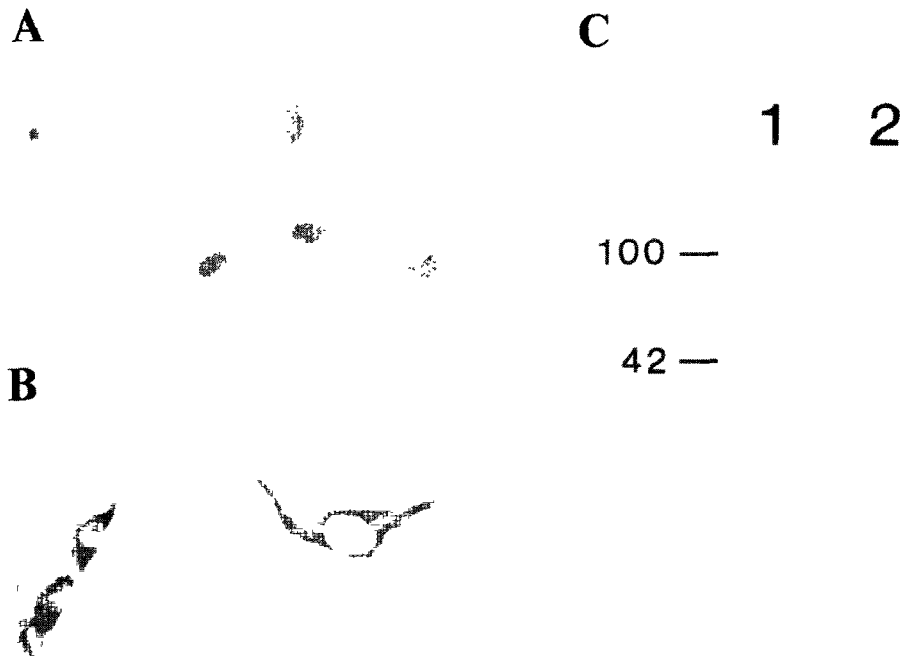

Fig. 2 Evaluation of rabbit anti-iNOS antiserum. (A) Immunocytochemistry with non-stimulated endothelial cells The cytoplasm around the recognizeable nuclei is not stained. (B) Immunocytochemistry with cytokine activated endothelial cells. Seen is an intense staining of the cytoplasm (C) Western blot analysis with lysate of (1) activated or (2) normal endothelial cells The molecular weight markers of 42 and 100 kDa are indicated The antiserum reacts with a protein of 130–140 kDa MW in lane 1.

Table I

Association of iNOS positivity with macrophage infiltration of islets

	No. of islets				
	Without infiltration		With infiltration		
	Total ($ED1^+$ cells ≤ 4)	iNOS pos.	ED1 pos.	iNOS pos.	ED1 pos. iNOS pos
Adult BB rats, diabetes-prone ($n = 4$)	26	0	47	46	46
Wistar rats ($n = 3$)	29	0	1	0	0

Staining with iNOS antibodies was found in areas of macrophage infiltration of islets from diabetes-prone BB rats (Fig. 3). Semiquantitative analysis showed macrophage infiltration in 47 of 73 islets in adult diabetes-prone BB rats versus 1 of 30 islets in age-matched Wistar rats (Table I). Nearly all islet areas with macrophage infiltration also stained for iNOS while in no case iNOS protein was demonstrated in islets in the absence of $ED1^+$ cells (Table I).

4. DISCUSSION

The data presented demonstrate the presence of iNOS gene transcripts and of iNOS protein in pancreatic lesions of adult diabetes-prone BB rats. The expression of the iNOS gene is disease-associated, iNOS mRNA was not detected in pancreata of normal Wistar rats, of young diabetes-prone BB rats prior to the onset of recognizable insulitis and of diabetes-resistant BB rats. Recently, a first study of iNOS mRNA expression in experimentally induced autoimmunity showed gene expression in the brain of rats after immunization with myelin basic protein [15]. Thus, expression of iNOS in target organs occurs in both, spontaneous and experimental autoimmunity. In the present study, we made use of a specific antiserum to identify the site of iNOS expression.

Immunohistochemistry showed a clear association of iNOS with islet areas of macrophage infiltration. In no case iNOS was found in the absence of ED1-positive cells. This indicates that macrophages themselves express iNOS. In addition, macrophages may have induced adjacent endothelial or endocrine islet cells by inflammatory mediators to secrete NO. Such mediators are IL-1 and TNFα, expression of these genes has been shown in inflamed islets of BB rats [16]. It has also been shown that highly purified β islet cells are able to produce NO upon challenge with cytokines [17]. Whether iNOS expression by endocrine cells occurs in vivo is not known. The present data indicate that the possible contribution of β cell iNOS is restricted to the close vicinity of macrophages. The other possible site of iNOS expression is the islet endothelium. In fact, recent studies have shown that islet endothelial cell cultures respond to cytokine challenge with sustained NO release [18].

In any case there is a striking association of iNOS expression with a local infiltrate of ED1 positive macrophages, which indicates a contribution of inflammatory macrophages to iNOS induction and expression. In this context it is of interest that macrophages from various organs of diabetes-prone BB rats respond to proper stimuli with excessive release of TNFα and oxygen radicals [19,20]. This inflammatory hyperreactivity of BB rat macrophages may promote iNOS expression at sites of macrophage infiltration.

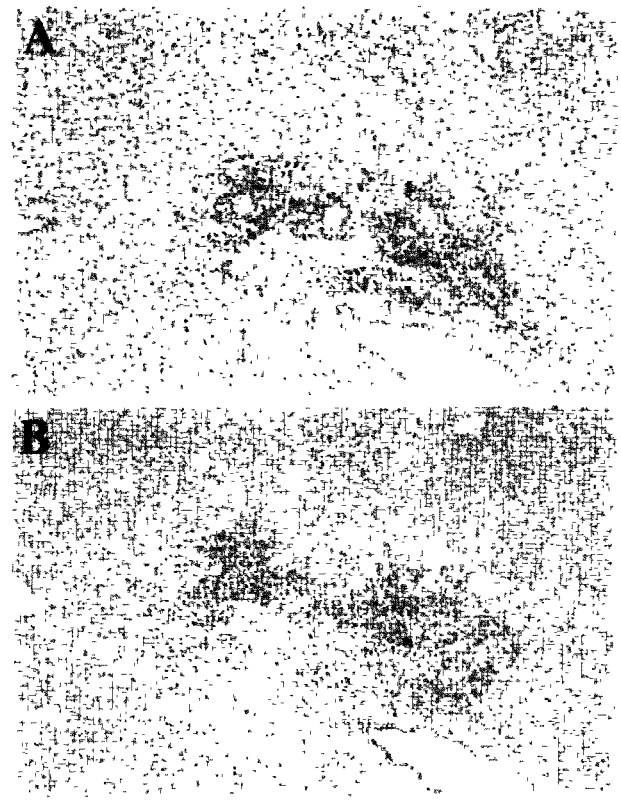

Fig. 3. Immunocytochemistry of pancreatic sections. (A) Section of a 10-week-old diabetes-prone BB rat stained for ED1. (B) The adjacent section stained for iNOS. Both antigens show the same pattern of distribution Islet areas negative for iNOS do not show ED1 staining

In summary, we show here for the first time the enhanced transcription and translation of iNOS in areas of inflammation during spontaneous autoimmunity.

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