The Intrathymic Expression of Insulin-related Genes: Implications for Pathophysiology and Prevention of Type 1 Diabetes

Vincent Geenen*, Pierre J. Lefebvre

Department of Medicine and Institute of Pathology, University of Liège, Liège, Belgium

Summary. Recent experimental work has challenged and shattered the old concept of a sequestration of pancreatic islet antigens from developing T-cells within the thymic environment. There is now compelling evidence that the central immunological tolerance of the whole insulin family may be induced during the process of T-cell ontogeny in the thymus. Transcripts of insulin-like growth factor II (IGF-II), IGF-I and insulin genes have been characterized in human, rat and mouse thymuses. At the peptide level, IGF-II was shown to be the dominant polypeptide of the insulin family in the thymus from different species. Data are presented which support a dual role of thymic IGF-II both in T-cell development as well as in T-cell negative selection. Using animal models of autoimmune diabetes, current research is investigating the hypothesis that a defect of thymic T-cell education to the insulin family is implicated in the pathophysiology of human Type 1 diabetes. An efficient and secure prevention of Type 1 diabetes could be designed on the basis of the strong natural tolerogenic properties of the thymus. © 1998 John Wiley & Sons, Ltd.

1. INTRODUCTION

A fundamental property of the immune system is its inability to react against the molecular structure of its host. In other words, before reacting against "non-self" infectious agents, the immune system must be tolerized to the "self". The induction of immune self-tolerance is a multi-step process which begins in the thymus during fetal development (central tolerance) and which also involves inactivating or anergizing mechanisms outside the thymus (peripheral tolerance).[1] It is well established today that the thymus is the primary lymphoid organ involved in the development of competent and self-tolerant T-cells.[2] From a histophysiological point of view, three types of cells have been described as constituents of the thymic environment which control T-cell differentiation. The predominant stromal cells (around 85%) are epithelial cells (TEC) which derive from embryonic endoderm of the 3rd and 4th

pharyngeal pouches. The contribution to the thymic epithelium of the ectoderm from the 3rd branchial cleft is still a matter of discussion. Among TEC of the outer cortex, thymic "nurse" cells (TNC) provide a very specialized micro-environment into which 50–150 immature T-cells migrate and differentiate at contact of these large epithelial cells.[3,4] Bone marrow-derived dendritic cells and macrophages represent the remaining components of thymic parenchyme. They are dedicated professional antigen-presenting cells although this property is also shared by TEC/TNC.[5] The dual physio-logical role of the thymus in T-cell development and in the negative selection of self-reactive T-cells (by clonal deletion or developmental arrest) remains a paradox hardly explained by the available models. Indeed, it is problematic to understand how two fundamental processes such as T-cell life and death can be mediated through a unique trimolecular complex: the self-antigen, the major histocompatibility com-plex (MHC)-encoded presenting protein and the T-cell receptor for the antigen (TCR).[6,7] On the basis that TEC/TNC from different species express a repertoire of neuroendocrine-related polypeptides, we have proposed a model which recapitulates at the molecular level the dual physiological role of the thymus. Thymic neuroendocrine-related precursors may engage two distinct types of interactions with differen-tiating T-cells.[8,9] These precursors are a source of *cryptocrine signals* which are targetted to the outer surface of TEC/TNC plasma membrane from where they can bind to neuroendocrine-type receptors expressed by contacting thymo-cytes (pre-T-cells). The same precursors can be processed into *neuroendocrine self-antigens* which are presented by the thymic MHC system.[10,11] It is thought that the central T-cell tolerance of neuroendocrine families could be mediated in this way. Neuroendocrine self-antigens usually correspond to peptide sequences which are highly conserved throughout the evolution of their family, and there is experimental evi-dence, at least for some of them, that their thymic presentation is not restricted in an allelic fashion.[12,13]

2. HISTORICAL OVERVIEW OF THYMIC INSULIN-LIKE POLYPEPTIDES

For a long time, an unbreakable frontier was thought to separate pancreatic insulin-secreting islet β-cells and the developing T-lymphocytes within the thymus. A series of investigations have demonstrated that such a concept is no longer valid and that the T-cell system may be educated during thymic ontogeny to recognize the molecular identity of the pancreatic islet β-cell, i.e. the endocrine secretion of insulin.

Already by 1965, on the basis that AKR female mice develop hypoglycaemia and thy-mic hyperplasia associated with lymphatic leu-kaemia, Pansky *et al.* reported the presence of an insulin-like peptide within the thymus from AKR strain mice, as well as from bovine and porcine species.[14] Mouse thymic extracts induced a marked hypoglycaemic effect when subcutaneously injected into young (3–4 weeks of age) female AKR mice. The biological action of thymic extracts even exceeded that of simi-larly prepared pancreatic extracts. Important quantities of immunoreactive (IR) insulin (from 150 to >500 μU/g) could be extracted from the thymus, in particular with acid–alcohol from beef and pork thymus. Using fluorescein-labelled guinea-pig anti-beef insulin, fluor-escing elements were observed among reticular cells and in Hassall's corpuscles of pig thymus. Interestingly, the authors discussed the agree-ment of their data with previous studies reporting hypoglycaemia associated with mass-ive tumours of epithelial and mesodermal ori-gin.[15,16] They also postulated a role for this thymic insulin-like reticular hormone in the initiation of immunological competence and lymphopoiesis.

To our knowledge, this thymic insulin-like reticular biological and IR peptide has not been further characterized. In 1987, however, Han *et al.* described a wide distribution in human fetal tissues of insulin-like growth factor (IGF) mess-enger RNA (mRNA) by *in situ* hybridization with oligonucleotides complementary to por-tions of human IGF-I and IGF-II.[17] In the fetal thymus, IGF transcripts were found in the cap-sule and in interlobular septa with a relative predominance of IGF-II transcripts. No IGF mRNAs could be evidenced in thymocytes. It has to be noted, however, that other authors failed to detect IGF-I mRNA in human fetal tissues.[18] Through the use of immunocytochem-istry, IGF-IR-positive cells were found in the corpuscles and interlobular septa of human fetal thymus.[19] The positive cells were described as having the morphology of imma-

ture haematopoietic precursors. Since the antibodies recognized both IGF-I and IGF-II, the identification of specific IGFs was precluded. The relative quantification of IGF transcripts in the human fetal thymus revealed two-fold more IGF-II (±25 pg/10 μg poly A$^+$ RNA) than IGF-I mRNAs (±12 pg/10 μg poly A$^+$ RNA),[20] suggesting that IGF-II is synthesized in greater quantities than IGF-I in this organ during embryonic development.

Since central T-cell tolerance of neuroendocrine functions could be induced by the thymic repertoire of neuroendocrine self-antigens,[21] a series of investigations was initiated in order to identify the dominant member of the insulin family expressed in the thymic microenvironment. Using a panel of specific antibodies directed against several members and epitopes of the insulin family, IR IGF-II was clearly identified within TEC of the subcapsular cortex and the medulla of human and rat thymus.[22] A monoclonal antibody (mAb) against human proinsulin[23] revealed a slight labelling outside thymic lobules, in the interstitial tissue of interlobular septa and the thymic capsule. The staining with mAbs to the C-terminal part of the bovine insulin B chain (OXI-002 and OXI-005, Novo Nordisk) was mainly negative within the thymic stroma, while a slight labelling could be observed in connective components. Some IGF-I-positive cells were also identified in thymic lobules but these cells were not epithelial; their distribution and morphology were similar to macrophages. Further characterization of thymic IR IGF-II confirmed that ±65% of the immunoreactivity corresponds to recombinant human IGF-II. IR IGF-II was not detected in the supernatant of primary cultures of human TEC, either in basal conditions or after treatment with epidermal growth factor. However, a large part part of IR IGF-II was shown to be associated with TEC membranes. Using specific radioimmunoassays, IGF-II was also shown to be the dominant thymic peptide of the insulin family. In the human thymus, IGF-II, IGF-I and insulin concentrations were respectively 96.7 ± 10.6 ng/g, 42.9 ± 5.0 ng/g and <0.1 ng/g wet weight. Using confocal microscopy and immunocytochemistry with specific antibodies, IGF-II (but neither IGF-I nor insulin) was detected at the outer surface of TEC plasma membrane (Achour et al., submitted). IGF-II transcripts

have been isolated from whole human thymic extracts, as well as from primary cultures of human TEC. Through the use of polymerase chain reaction (PCR) with different primers, the intrathymic expression of the IGF-II gene was found to be under the control of the same promoters as in other fetal and adult extrahepatic tissues.[24,25]

The effects of IGF-II overexpression were investigated by the generation of transgenic mice carrying one of three different human IGF-II minigenes in which different non-coding exons (exon 5, truncated exon 5 or exon 6) preceded the coding exons 7, 8 and 9. The three IGF-II minigenes were placed under the control of the MHC H-2K^b promoter. The highest levels of IGF-II minigene mRNA expression were detected in lines containing the truncated exon 5 construct. The highest mRNA expression was found in thymus and spleen; serum IGF-II levels at 8 weeks of age were increased seven- to eight-fold in homozygous transgenic lines but no significant hypoglycaemia was observed. Among several organs, only the thymus showed a significant increase of weight in these transgenics, in agreement with the high mRNA expression within this organ.[26] With in situ hybridization, expression of the IGF-II transgene was found with high intensity in the thymic medulla and in the white pulp/marginal zone of the spleen, whereas there were scattered positive cells in the thymic cortex and in the splenic red pulp. Hybridization was restricted to non-lymphocytic cells and immunocytochemistry revealed intense IGF-II peptide staining with the same distribution of IGF-II mRNA.[27]

Going back to the initial observations made by Pansky et al,[14] there is now ample evidence that the thymic insulin-like reticular factor corresponds in fact to IGF-II. The IGF-II structure closely related to (pro)insulin might explain a cross-reactivity with the antibodies against insulin used at that time. The hypoglycaemic properties of IGF-II have been well described[28] and might also explain the biological activity of thymic extracts on glucose metabolism. The syndrome of hypoglycaemia and lymphatic leukaemia associated with thymic hyperplasia of some AKR female mice could result from an overexpression of Igf2 in hyperplasic thymic epithelium, a subsequent secretion of IGF-II

in the bloodstream, as well as a profoundly disturbed intrathymic lymphopoiesis.

The hypothesis for a central T-cell tolerance of the peripheral pancreatic islets was further supported by the observation that transcripts of insulin, glucagon, somatostatin, pancreatic polypeptide and the 67 kDa isoform of glutamic acid decarboxylase could be detected in the murine thymus using PCR with 30 cycles of amplification.[29] Thymic insulin gene (*INS*) expression was strongest in perinatal mice and persisted until 12 weeks of age. Two recent papers confirmed these findings and reported that *INS* transcripts, as well as (pro)insulin protein, are detected at low levels in the human fetal thymus. Thymic concentrations of insulin IR were 98–1200 fmol/g wet weight in the first study,[30] whereas proinsulin concentrations were 1.904 ± 1.428 SD pmol/mg protein and those of insulin were 0.437 ± 0.218 SD pmol/mg protein in the second study.[31] Since long class III variable number tandem repeat (VNTR) alleles (140–210 repeats) are associated with two- to three-fold higher thymic *INS* mRNA levels, the authors suggested that the protective effect of these alleles against Type 1 diabetes could be explained by the higher level of thymic *INS* expression, thereby facilitating the induction of central immunological self-tolerance of insulin. Using *in situ* hybridization, *INS* transcripts have been detected in thymic dendritic cells although the protein could not be detected by immunocytochemistry (F. Homo-Delarche, personal communication). The induction of a strong tolerogenic action by thymic (pro)insulin may nevertheless be questioned. Thymic (pro)insulin concentrations are very low and thymic T-cell tolerance rapidly decreases for peptide concentrations below 10 nM.[32,33] In addition, a recent discussion was raised about the precise boundaries of the *IDDM2* locus of susceptibility to Type 1 diabetes which could also include two genes close to the *INS* VNTR, the *tyrosine hydroxylase* and *IGF2*.[34,35] There is also strong evidence that implicates an imprinted gene in the *INS-IGF2* region of chromosome 11p15 in the aetiology of Type 1 diabetes. Since *INS* is not imprinted in the human fetal pancreas, the monoallelic expression of *IGF2* in the thymus might explain why insulin-dependent diabetes is transmitted by diabetic fathers more than twice as often as by diabetic mothers.[36]

3. THE DUAL PHYSIOLOGICAL ROLE OF THYMIC IGF-II

A functional signalling mediated by IGF-II and IGF receptors is plausible between TEC/TNC and immature T-cells since type 1 and type 2 IGF receptors are detected in rat thymocytes and murine thymoma cells,[37] on human phytohaemagglutinin (PHA)-activated T-cells and on anti-CD3-activated human T-lymphocytes.[38,39] Kooijman *et al.* have described a differential expression of type 1 IGF receptors in relation to the stage of activation and differentiation of human T-lymphocytes.[40] Interestingly, in IGF-II transgenic mice the increased thymic cellularity is associated with a stimulated generation of phenotypically normal T-cells, in particular CD4^{+} T-cells.[41] In our hands, specific type 2 IGF receptors were found to be expressed by a murine immature T-cell line as well as by Jurkat T-cells.[42] By affinity cross-linking, the type 2 IGF receptor expressed by lymphocytes was shown to have a molecular mass (± 260 kDa) similar to that found on other cells (Goxe *et al.*, in preparation). On the basis of these observations, thymic IGF-II may thus intervene in a functional cryptocrine signalling between TEC and pre-T-lymphocytes during their intrathymic developmental programme.

As discussed earlier, IGF-II was found to be the dominant member of the insulin hormone family expressed by TEC/TNC from different species. Thymic IGF-II concentrations are high (± 100 ng/g) and it has been shown that the final orientation of an antigen-driven immune response, towards either priming (immunogenic) or tolerizing (tolerogenic), is determined by the dominant T-cell epitopes.[43] Thus, if a tolerogenic immune response follows the thymic expression of an insulin-related protein, this should firstly concern the dominant thymic peptide of the insulin family. Thymic IGF-II may induce a strong central immune tolerance of IGFs similar to that observed for peripheral proteins resulting from their thymic expression.[44] The crucial role of IGFs in fetal and postnatal development implies that these proteins have to be protected from an autoimmune attack. As an important though indirect argument for the tolerogenic properties of IGF-II, it is known that the production of specific antibodies is much more difficult with IGF-II than with insulin or IGF-I.[45,46] The IGF-

II molecule includes peptide sequences which are highly conserved throughout the evolution of the insulin family.[47] Because of this close homology, thymic IGF-II would be a good candidate for inducing central immune tolerance of the insulin family although the tolerance of insulin *per se* would be weaker. This might explain why B- and T-cell autoreactivity to insulin has been equally observed in diabetic and related non-diabetic individuals.[48]

4. RELATIONSHIPS BETWEEN THE THYMUS AND TYPE 1 DIABETES

The question of a defective thymic censorship as one element implicated in the pathophysiology of autoimmune diabetes has not been intensively investigated until now. Some elegant studies, however, have provided experimental arguments supporting this idea. Neonatal thymectomy prevents the emergence of diabetes in an animal model of Type 1 diabetes, the Bio-Breeding (BB) rat.[49] The benefit of thymectomy might in fact result from the removal of a defective organ responsible for the continual release and the peripheral enrichment of intolerant self-reactive T-lymphocytes. Conversely, the occurrence of diabetes is prevented by the transplantation of thymus from diabetes-resistant to diabetes-prone BB rats.[50] Transplantation of the thymus from NOD mice to

Table I. The dual role of thymic IGF-II in T-cell differentiation

Cryptocrine signalling	IGF-II or IGF-II-derived self-antigen presentation
Physiology Accessory pathway in T-cell development	*Physiology* Central T-cell tolerance of the insulin family and islet β-cells
Pathophysiology T-cell lymphomas Paraneoplasic hypoglycaemia	*Pathophysiology* Type 1 diabetes
Pharmacology Immunomodulation by IGF agonists and antagonists	*Pharmacology* Tolerogenic vaccination

diabetes-resistant mouse strains was also shown to induce diabetes in the recipients.[51] While bone marrow transplantation is ineffective in autoimmune diseases of MRL/+ mice, thymus transplantation was found to be a crucial factor for their prevention.[52] An abnormal process of thymic T-cell selection has been suggested on the basis that diabetes-resistant BB rat thymus contains thymocytes predisposed to autoreactivity.[53] As another argument for a role of thymic T-cell repertoire selection in the pathogenesis of autoimmune diabetes, grafts of pure thymic epithelium from NOD mouse embryos to newborn C57BL/6 athymic mice were shown to induce CD4 and CD8 T-cell-mediated insulitis and sialitis.[54] At the histological level, a defect in thymic censorship could result from a disorganization in the tissue microenvironment such as the giant perivascular spaces observed in the NOD mouse thymus[55] and the epithelial defects of BB rat thymus.[56] Altogether these observations support the hypothesis that a defective thymic induction of central T-cell tolerance plays a role in the pathophysiology of Type 1 diabetes. IGF IR elution profiles on G75 columns were analysed in thymic extracts from Wistar Furth (WF), diabetes-resistant and diabetes-prone BB rats. A peak of >10 ng/ml was observed in the elution profile of WF thymus extract; a peak around 1.5 ng/ml was eluted from diabetes-resistant BB rat thymus extract, while IGF-II concentrations were almost undetectable in the thymus from diabetes-prone BB rats.[57] These observations strongly suggest at the molecular level that a defect in thymic IGF-II-mediated T-cell education might be implicated in the pathogenesis of autoimmune insulin-dependent diabetes.

5. TOWARDS A TOLEROGENIC FORM OF VACCINATION FOR THE PREVENTION OF TYPE 1 DIABETES?

Three factors are thought to be implicated in the pathophysiology of Type 1 diabetes. (1) The effector components are CD4 and CD8 autoreactive T-cells which are specifically oriented against the pancreatic islet insulin-secreting β-cells. These autoreactive T-cells could result from a spontaneous breakdown of T-cell tolerance, either at the central and/or the

peripheral level. (2) A series of extra- and intra-MHC genes have been demonstrated to be related to Type 1 diabetes. Some of these genes probably intervene in the presentation of islet autoantigens to autoreactive T-lymphocytes, but others certainly not. (3) An environmental factor is finally involved and could establish a pathogenetic link between pancreatic islet auto-antigens and autoreactive T-cells. The hypothesis of a molecular mimicry between islet auto-antigens and microorganisms might intervene at this level and has recently received an interesting experimental support.[58] The intervention of microbial superantigens has also been proposed to account for the activation of peripheral autoreactive T-cells.[59] A preventive strategy of Type 1 diabetes can hardly be designed on the basis of the genetic components of the disease or the hazardous external factors. Manipulation of autoreactive T-cells seems to be a more promising way by which an efficient prevention could be designed.

There is now considerable evidence that insulin is one among other autoantigens tackled by various autoreactive components of the immune system in both animal and human Type 1 diabetes.[60,61] Moreover, insulin is a specific marker of the islet β-cells. Oral, intranasal and parenteral administration of insulin or insulin-derived dominant autoantigens have been shown to inhibit the occurrence of diabetes in animal models of Type 1 diabetes.[62,63] However, one cannot exclude the risk of triggering autoimmunity by peripheral administration of an autoantigen.[64] Reprogramming the immune tolerance which is thought to be broken in autoimmunity represents an attractive srategy for the prevention of devastating autoimmune diseases such as Type 1 diabetes. Such reprogramming could be based on the natural tolerogenic properties of the thymic epithelium. Instead of a classical "vaccination" (with immune activation and induction of "memory" immunocompetent cells), a form of "tolerogenic" vaccination is proposed that would lead to the deletion or the anergy of peripheral autoreactive T-cells. The induction of T-cell tolerance following peptide vaccination has already been obtained with synthetic peptides representing cytotoxic CD8 epitopes of T-cells oriented against tumours or viruses.[65] An efficient and non-toxic prevention of Type 1 diabetes, perhaps even its definitive eradication,

could depend upon the strategic choice that will be made between either the induction of tolerance to β-cell-specific autoantigens (such as insulin, glutamic acid decarboxylase, or others) following their parenteral administration, or the exploitation of the natural tolerogenic properties of thymic IGF-II, the self-antigen precursor of the insulin hormone family.

Acknowledgements

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