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Evidence that insulin-like growth factor 2 (IGF2) is the dominant thymic peptide of the insulin superfamily

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Abstract. The central T-cell tolerance of neuroendocrine functions has been proposed to be primarily induced by the thymic repertoire of neuroendocrine self antigens. The present study aimed at characterizing the human thymic insulin-related autoantigen able to represent the pancreatic B-cell function in face of the developing T-cells. Immunofluorescence studies were performed on human and rat thymic sections, as well as on the rat IT-45R1 thymic epithelial cell line using several antibodies to epitopes of the insulin peptide superfamily. These studies identify beyond any doubt that insulin-like growth factor 2 (IGF2) is the dominant thymic peptide of the insulin family. The sequence of an insulin-derived autoantigen is proposed. This autoantigen is a nonamer and has a hydrophobic residue leucine (L) at position 9. In the human species, this autoantigen would primarily be tolerogenic for the pancreatic B-cell endocrine function during fetal development.

Introduction

There is now ample evidence that type I diabetes or insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease which is mediated by a specific cytotoxic aggression of T-cells against the insulin-secreting B-cells of pancreatic islets of Langerhans [1]. The differentiation of T-cells occurs primarily in the thymus and, on the basis of our previous studies about the thymic repertoire of neuroendocrine self antigens, we have described a model which transposes at the peptide level the dual role of this lymphoid organ in positive and negative selection of T-cells [2–5]. Briefly, according to that model, neuroendocrine-related peptides from thymic epithelium could mediate a cryptocrine-type cell-to-cell signalling by interacting with their cognate receptors expressed by the target T-cells [6]. As a consequence, the distinct accessory

pathways generated by the thymic environmental neuropeptides would be involved in the process of T-cell positive selection. Because of their close amino acid homologies with the hormonal members of their respective families, thymic neuroendocrine-related polypeptides might also act as self antigens representing their related neuroendocrine family and facing T-cells along their differentiation. In that latter case, the process could lead to the negative selection of potential autoreactive T-cells emerging during the random recombination of the genes coding for the chains of the T-cell receptor for antigen (TCR). In this research perspective, the most striking example of the intimate dialogue between the neuroendocrine and immune systems during T-cell differentiation was provided by our demonstration that the epithelial component of thymic 'nurse' cells (TNCs) [7] express a neuroendocrine-like phenotype [8]. We have already shown that our model can be applied to the neurohypophysial and tachykinin peptide superfamilies [9, 10] and is in close accordance with the recognized functions of TNC/TEC [11–13]. The main objective of this preliminary study was to investigate the application of our model to the insulin family.

Insulin is the hormonal member of a superfamily of peptides also including somatomedins or insulin-like growth factors (IGFs) 1 and 2, relaxin, insect prothoracicotrophic hormone and other insulin-related peptides detected in low species [14–16]. IGF1 is a 70-amino acid basic polypeptide that mediates most of the growth-promoting actions of growth hormone. The function of IGF2, a 67-amino acid polypeptide, is still largely unknown but it is acknowledged to play its major role in fetal development, especially in the central nervous system [17]. The first report on an insulin-like thymic factor was published in 1965 by Pansky and coworkers [18]. To our knowledge, their observation was not further investigated until 1987, when Han et al. described the expression of IGF genes within various human fetal tissues [19, 20]. However, these authors clearly stated that their experimental tools did not allow them to discriminate neither the cellular source of thymic IGF mRNAs/peptides, nor their precise biochemical nature [20]. Consequently, the identification and the localization of IGF-related peptides within the human thymus was not conclusive. We decided to further investigate that specific point because of its potential implication in the induction of central T-cell tolerance of pancreatic endocrine function.

Materials and methods

Antibodies. One monoclonal antibody (mAb) to human proinsulin (S1 7B7F9) was provided by Dr J.C. Sodoyez (University Hospital of Liège-Sart Tilman, Belgium) [21]; its dominant epitope includes the residue His (H) in position 10 of the insulin B chain [22].

Rabbit polyclonal antibody to rat IGF2 was purchased (Biogenesis Ltd., lot No. 910812J, UK) and was used at the dilution 1:20. Cross-reactivity was 5% with human IGF1, 1% with human insulin, and 0% with atrial natriuretic peptide, oxytocin, human adrenocorticotropin (1–39), human calcitonin, and vasoactive intestinal peptide.

Rabbit polyclonal antibody to human IGF1 was also obtained from Biogenesis Ltd. (UK) and was used at the dilution 1:10. Cross-reactivity was 100% with human, bovine and ovine IGF1, and very weak with human and human or rat IGF2 (< 1%).

Two mAbs to bovine insulin were graciously obtained from Novo Nordisk. The antigenic epitopes of OXI-002 (IgG1, kappa) and OXI-005 (IgG1, kappa) were located in the C-terminal part of the bovine insulin B-chain. Both mAbs were used at the initial dilution of 1:10. Cross-reactivity of OXI-002 was 50% with human and porcine insulin, and 1% with bovine proinsulin. Cross-reactivity of OXI-005 was 33% with porcine insulin, 3% with human insulin and less than 1% with bovine proinsulin.

O33 (used at the dilution 1:50) is a mAb that recognizes one of the dominant epitopes of the neurohypophysial peptide family and that labels TEC from the subcapsular cortex and the medulla [5].

The mAb A2B5 (ATCC HB-29; dilution 1:1) recognizes a complex GQ ganglioside expressed on the membranes of type I astrocyte precursors, neural crest-derived cells, and cell populations belonging to the diffuse neuroendocrine system (like pancreatic islet B-cells) [23]. A specific immunolabelling of the epithelial component of TNC and of TEC from the subcapsular cortex and medulla has been previously described in detail [8, 24].

We used rabbit polyclonal antibodies to human cytokeratins (Immunotech, France) as well as the anti-keratin mAb KL4 (1:100) [25].

All mAbs shared an IgG1 isotype except O33 and A2B5 that were typed as IgM.

Thymic samples. Thymic fragments were obtained from children (aged 1 month to 5 years) who were undergoing cardiac corrective surgery for congenital cardiopathies. Rat thymuses were excised from Wistar rats (around 6 weeks old). The fragments were embedded in Tissuetek, frozen on dry ice and stored at −70 °C. Frozen sections (5μm) were cut on a cryostat, air dried, and post-fixed with picric acid/formaldehyde as previously described [26].

Cultures of rat thymic epithelial cell line IT-45R1. The rat TEC line IT-45R1 [27] was developed and kindly provided by Dr T. Itoh (Tohuku University, Japan). Cells were cultured in RPMI 1640 medium (Flow) supplemented with 10% heat-inactivated fetal calf serum, 2×10^{-3} M glutamine, 10^{-3} M sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Immunocytochemistry. Sections were rinsed in washing buffer (Tris-NaCl buffer 0.5 M, pH 7.6, containing 0.05% Tween 20 and 0.02% NaN_3). They were preincubated for 30 min at room temperature with 10% normal goat serum (NGS). The antibodies were diluted in 0.1 M Tris-HCl, pH 7.6, supplemented with 0.02% NaN_3. First-step antibodies were incubated overnight at +4 °C (anti-insulin-related peptides, O33) or 30 min at room temperature.

After rinsing in washing buffer, fluorescein-conjugated goat antisera to rabbit Ig, or to mouse IgG1 or IgM (Fc specific), diluted 1:100 and 1:40, were used as second-step reagent for 30 min at room temperature. After three washings, the sections were mounted with glycerol/gelatin medium.

Fluorescence of tissue sections was observed with a Zeiss fluorescence microscope (Axioskop 20), equipped with a photomicrographic system MC 100, and using × 10, × 20 or × 40 objectives. Photographs were taken with Kodak Tri-X (400 ASA) and automatically-determined exposure times varied between 9 and 50 s.

Controls. Control experiments included dilution buffer, normal rabbit serum, and control ascites in place of the first-step antibodies. Preadsorption of anti-IGF2 and anti-IGF1 was made with the corresponding antigens (50 µM) coupled to CNBr-activated Sepharose-4B (Pharmacia, Sweden).

Results

Distribution of insulin-related peptides in the human and rat thymus. The polyclonal antibodies to rat IGF2 revealed a marked immunolabelling of stromal cells in the subcapsular cortex and in the medulla of human thymus (Fig. 1A). This distribution was quite similar, although not strictly superimposible, to the staining obtained with anti-keratin KL4 MAb (Fig. 1B). With anti-human proinsulin mAb, a slight labelling could be observed outside thymic lobules, in the interstitial tissue of the thymic capsule and interlobular septae (Fig. 1C). Similarly, the staining with anti-insulin mAb OXI-002 was negative within the thymic stroma, while a slight labelling of connective components could be observed (Fig. 1D). Identical results were obtained with anti-insulin mAb OXI-005 (data not shown). In rat thymic sections, anti-rat IGF2 antibodies sharply labelled stromal cells that were more dispersed throughout the thymic parenchyme and that displayed an epithelial-like morphology (Fig. 1E). With anti-human IGF1, an immunostaining can be observed in an interlobular septum, as well as in a few cells within the thymic lobule, but without any epithelial-like appearance (Fig. 1F). We observed an identical pattern of immunostaining in human thymic sections using the same anti-IGF1. Solid-phase absorption of antibodies with their respective antigens resulted in significant

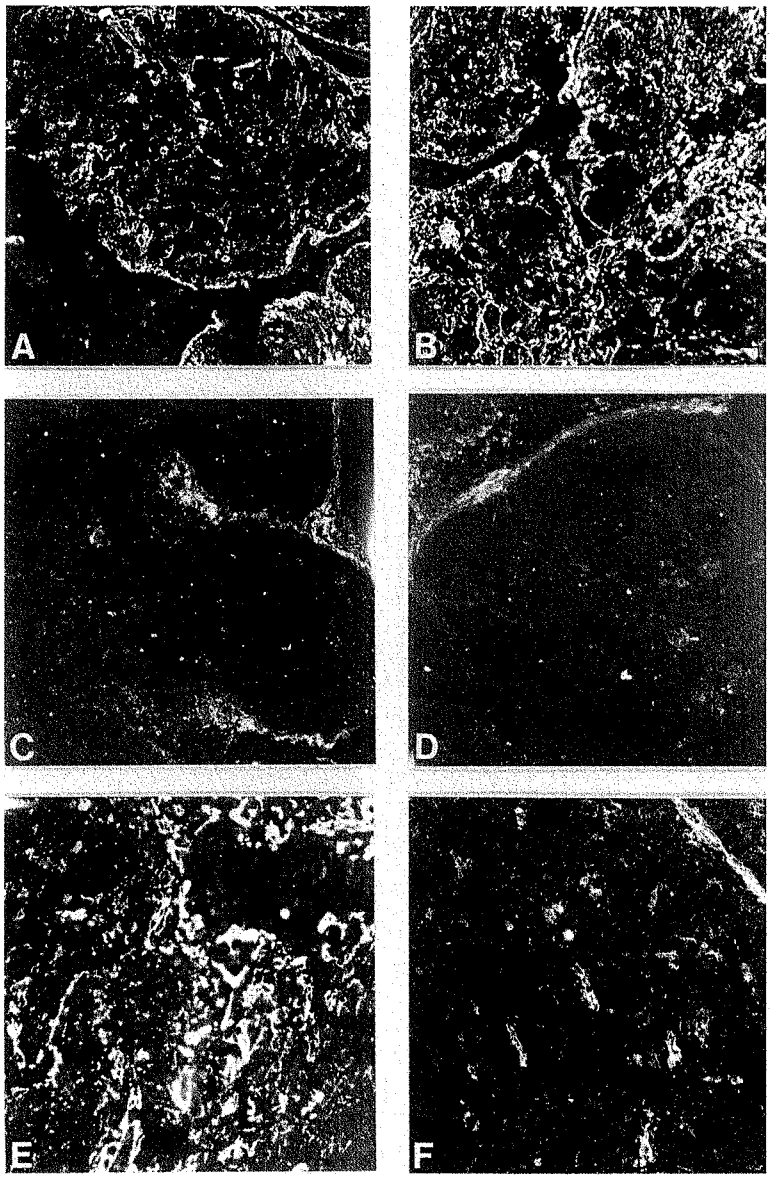

Fig. 1. Identification and localization of IGF2 in human (A–D) and rat (E, F) thymic sections. Immunofluorescence labelling through the use of: (A) rabbit polyclonal Ab to rat IGF2 (1:20) and FITC goat anti-rabbit Ig (1:100); (B) anti-keratin mAb KL4 (1:100) and FITC goat anti-mouse IgG1 (1:40); (C) anti-human proinsulin mAb S1 7B7F9 (1:1) and FITC goat anti-mouse IgG1 (1:40); (D) anti-bovine insulin B chain mAb OXI-002 (1:10) and FITC goat anti-mouse IgG1 (1:40); (E) rabbit anti-rat IGF2 polyclonal Ab (1:20) and FITC goat anti-rabbit Ig (1:100) and (F) rabbit anti-human IGF1 polyclonal Ab (1:10) and FITC goat anti-rabbit Ig (1:100).

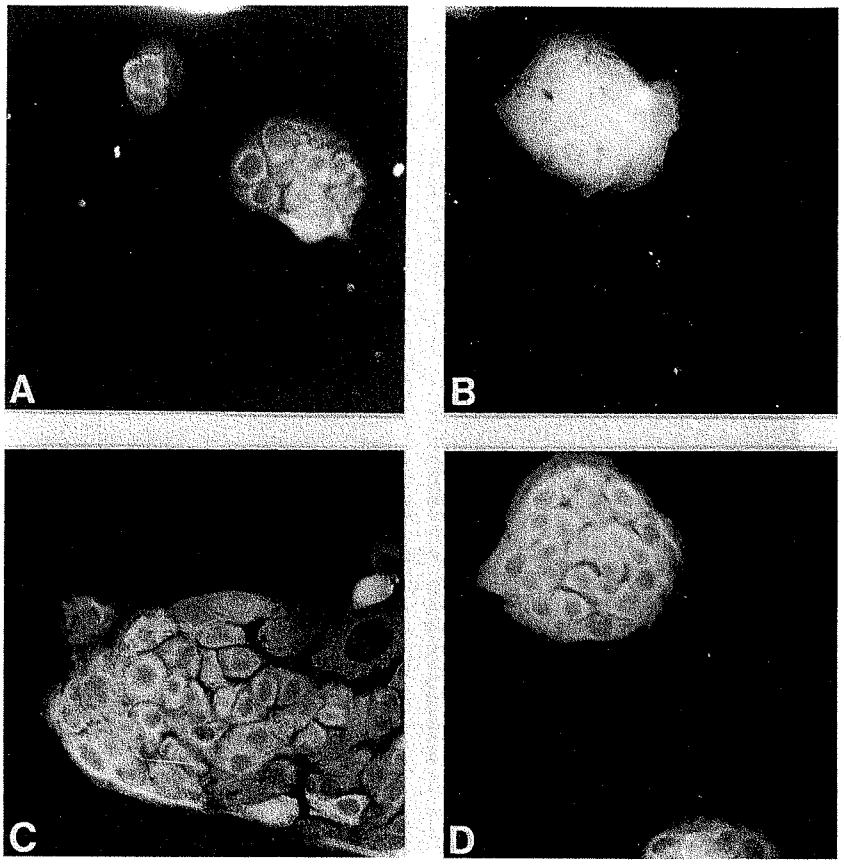

Fig. 2. 4 day-cultured rat IT-45R1 thymic epithelial cells immunostained with: (A) rabbit anti-human IGF1 (1:10) and FITC goat anti-rabbit Ig (1:100); (B) rabbit anti-rat IGF2 (1:20) and FITC goat anti-rabbit Ig (1:100); (C) mAb A2B5 (1:1) and FITC goat anti-mouse IgM (1:40); (D) mAb O33 (1:1) and FITC goat anti-mouse IgM (1:40).

decrease of the labelling with anti-IGF2, while only a weak fainting was obtained with anti-IGF1 (data not shown).

Immunocytochemical analyses of cultured rat IT-45R1 TEC. In rat IT-45R1 TEC cultures, a weak staining of cell cytoplasm was obtained with anti-human IGF1 (Fig. 2A); this was in marked contrast with the intense labelling obtained with anti-rat IGF2 antibodies (Fig. 2B). It is also important to note that the exposure time was 45 s for IGF1, and 10 s for IGF2. Cultured rat TEC could also be immunostained with A2B5 and O33 MAbs (Fig. 2C & 2D). The different labellings displayed a filamentous shape in the cytoplasm of TEC. No significant immunostaining of rat IT-45R1 TEC could be observed with the other antibodies.

Discussion

Our immunolocytochemical data identify beyond any doubt IGF2 as the dominant member of the insulin peptide family which is expressed by epithelial cells from the subcapsular cortex and the medulla in human and rat thymus. This conclusion is further attested by the cytoplasmic staining of cultured rat IT-45R1 thymic epithelial cells. The labelling with anti-rat IGF2 was intense, whereas that obtained with anti-IGF1 was weaker and the corresponding photographic impression required four fold longer exposure times. Under our experimental conditions, it is however impossible to definitively exclude a potential IGF1 expression by thymic epithelium but, if it was the case, IGF1 would be expressed at a very much lower rate. As shown in Fig. 1F, in situ synthesis of IGF1 could also occur within thymic macrophage-like cells. The negative labelling with mAb to human proinsulin can be easily explained by the absence of residue His (H) in the corresponding region of IGF2. The negative results obtained with anti-insulin OXI-002 and OXI-005 can also be explained by the fact that the C-terminal of insulin B chain is quite different from its counterpart in IGF2. Although the classical limitations of immunocytochemical analyses have to be applied to our data, the present results are in close accordance with previous studies which have evidenced the expression of IGF genes within human fetal organs [19, 20]. In our hands, thymic IGF2 immunoreactivity has been found in TEC from the subcapsular cortex and the medulla of the human thymus, a distribution which had already been described for other neuroendocrine markers such as A2B5 and O33 (one of the immunodominant epitopes of the neurohypophysial family) [26]. Since outer cortex-derived TNCs were also demonstrated to be labelled with A2B5 and O33 MAbs [8], it is most plausible that their epithelial component also expresses IGF2. The major implication which can be drawn from our observations is the fact that a significant part of the human thymic epithelium is the site for the expression of antigens related to the insulin family. This constitutes an additional experimental support to our working model on the 'education' of the T-cell system in neuroendocine principles that is exerted through the thymic repertoire of neuroendocrine self peptides [28].

As it can be observed in Fig. 2, the filamentous immunolabelling of cultured rat TEC suggests an intimate relationship between the IGF2 secretory process and the intermediate filaments of the cytoskeleton. It is actually more and more admitted that various growth factors are using a secretory pathway different from the classical one through Golgi apparatus-derived secretory granules. In this special class of factors without hydrophobic signal sequences, one can find IGFs, interleukin-1, basic and acidic fibroblast growth factors, platelet-derived growth factor, and thymosin-related peptides. Analogous mechanisms are also proposed for the translocation of peptides into the endoplasmic reticulum for

presentation by MHC molecules [29]. Our data suggest that the intermediate filaments of TEC could actively intervene in these novel secretory pathways.

We have previously proposed a dual physiological role for the thymic peptide repertoire which was founded on the expression of neurohypophysial- and tachykinin-related self peptides in the thymus [30]. This model also applies to thymic IGF2. A functional intercellular signalling through IGF-related signals and receptors is very plausible between TEC and pre-T-cells since type 1 and 2 IGF receptors have been shown to be expressed in the thymus, on human phytohemagglutinin-activated T lymphocytes and on anti-CD3-activated human T lymphocytes [31, 32]. These findings strongly support that thymic IGF signals (originating from TEC) and receptors (expressed by thymocytes) might intervene in T-cell differentiation and positive selection [33]. A disturbance in this IGF-mediated cell-to-cell signalling could be involved in lymphoproliferative disorders as previously reported [34]. On the other hand, because of its high homology with the A and B domains of human proinsulin, thymic IGF2 could also represent the whole insulin peptide family facing the differentiating T-cell system during fetal development.

This latter point is highly significant with regard to the intrathymic mechanisms underlying the central T-cell tolerance of the main pancreatic endocrine function, the secretion of insulin by islet B-cells. It is now well accepted that a key event in the process of T-cell tolerance is the intrathymic negative selection of self reactive T-cells that emerge during the random recombinant of the genes coding for the TCR chains [35, 36]. The negative selection is induced by either anergy or clonal deletion (by apoptosis) of self reactive T-cells, and usually follows the presentation of autoantigenic amino acid sequences by thymic molecules derived from the major histocompatibility complex (MHC). Several autoantigens have been proposed to intervene in the pathological process, and many of them have been characterized. None of them can be reasonably considered as the primary diabetogenic autoantigen, and the whole process is now supposed to follow a cascade of autoimmune events leading to the final destruction of the islet B-cell [1]. Since it is the most specific marker of the B-cell function, insulin has long been sought to be a major autoantigen of IDDM. Autoantibodies against insulin have been detected in the sera of diabetic patients, but their pathogenic role has been questioned since they were also detected in the serum of normal patients [22]. The autoreactivity of T-cells against insulin has been previously investigated but was also evidenced in related non-diabetic individuals [37]. Interestingly, the sequence of residues 7–15 of the bovine insulin B chain was recently identified as a target self antigen for H-2K^b-restricted cytotoxic T-cells [38]. This was the first preliminary evidence that a class I MHC-restricted, insulin peptide-specific cytotoxic response could be experimentally induced. The authors also suggested that the sequence 7–15 of insulin B chain could be immunodominant over peptide sequences

derived from insulin A chain. This sequence was also identical in the insulin molecule from different species and could then be considered as a self peptide. Interestingly, and in close relation with our own observations, this sequence is highly homologous in the human IGF2 structure since 5 amino acids (on a total of 9) are identical (55% homology). Therefore, one potential major autoantigen representing the pancreatic B-cell function could be very close to the following sequences (single-letter code):

Human IGF2 CGGELVDTL

Human insulin CGSHLVEAL

More specifically in the human species, this IGF2-derived sequence would be acting as a tolerogenic factor mainly during fetal life along the differentiation of the T-cell system. This of course does not exclude a similar role for other sequences derived either from IGF1, or from insulin A or B chains [39]. It was also recently reported that, once the autoimmune process initiated, cryptic determinants of an autoantigen may become immunogenic [40]. It is also important to underline that these sequences are fitting a number of criteria which have been delineated for peptide presentation by MHC class I molecules [41–43], in particular the length of the sequence and the hydrophobic residue Leu (L) at position 9. Therefore, if we prove in a next step that thymic IGF2-derived sequences are in fact presented by thymic MHC molecules, this could lead to the precise identification of the peptides able to induce the central T-cell tolerance of pancreatic islet B-cell function. The pathogenetic process observed in type I diabetes mellitus might in fact derive from a defect of intrathymic mechanisms leading to a breakdown in the central immune tolerance of pancreatic endocrine function. Our studies demonstrate that thymic IGF2 could be the primary source of self antigens representing the insulin family in front of the differentiating T-cell system. A failure in the educational programme of T-cells could lead to the breakdown of immune tolerance to pancreatic B-cells and the emergence of autoreactive T-cells against insulin-related autoantigens. This is further supported by the fact that the insulin-IGF2 region on chromosome 11p encodes a gene implicated in HLA-DR4-dependent diabetes susceptibility [44]. In that perspective, the target pancreatic B-cells should be able to present autoantigen(s) related to the insulin family. Interestingly, some authors have reported the presence of insulin-like material in the membrane of pancreatic islet B-cells [45, 46]. Such translocation of an insulin-related antigen in the membrane of islet B-cells could also explain, at least partially, the prevention of autoimmune diabetes by intrathymic islet transplantation in the neonatal BB rat [47].

It is also noteworthy that the expression of IGF2 by TEC would provide a continual exposure of one autoantigen to developing T-cells, a mechanism which has been shown to be a prerequisite for the maintenance of in vivo toler-

ance [48]. It will be also of high interest to firmly prove the presentation of IGF2-derived sequences by thymic MHC class I molecules since a defect in the antigen presentation by those protein complexes has been suggested in NOD mice, an animal model of IDDM [49]. Finally, the potential implication of thymic IGF2 in the induction of central T-cell tolerance to pancreatic islet B-cell function does not exclude the intervention of peripheral pathways as recently suggested by the suppression of diabetes in NOD mice by oral administration of porcine insulin [50].

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