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Journal of Neuroimmunology 76 (1997) 161–166

Journal of Neuroimmunology

Identification of neurotensin-related peptides in human thymic epithelial cell membranes and relationship with major histocompatibility complex class I molecules

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Received 17 September 1996; revised 28 January 1997; accepted 29 January 1997

Abstract

This study shows the expression at the cell surface of human thymic epithelial cells (TEC) of a neurotensin (NT)-like immunoreactivity. NT radio-immunoassay (RIA) revealed that cultured human TEC contain ± 5 ng immunoreactive (ir) NT/10^6 cells, of which 5% is associated with plasma cell membranes. HPLC analysis of NT-ir present in human TEC showed a major peak of NT-ir corresponding to NT_{1-13}. NT-ir was not detected in the supernatant of human TEC cultures. Using an affinity column prepared with a anti-MHC class I monoclonal antibody, NT-ir-related peptides were retained on the column and eluted together with MHC class I-related proteins. According to the elution time on HPLC of these peptides, they correspond to intact NT_{1-13}, as well as to smaller fragments of NT_{1-13}.

Keywords: Thymus; Neuroendocrine self-antigens; Neurotensin; T-cell self-education; Cryptocrine signaling

1. Introduction

The thymus is the primary lymphoid organ involved in the differentiation of competent and self-tolerant peripheral T-lymphocytes (Kruisbeek, 1993). The dual physiological role of the thymus in T-cell development and in the negative selection of self-reactive T-cells (by clonal apoptotic deletion or by developmental arrest) remains unexplained at the molecular level. It remains problematic to understand how two fundamental opposite events such as T-cell life and death could be mediated through an unique trimolecular complex made by the major histocompatibility complex (MHC) molecule, the self-antigen and the T-cell receptor for the antigen (TCR) (Allen, 1994; Ashton-Rickardt and Tonegawa, 1994).

Thymic epithelial and nurse cells (TEC/TNC) from different species express a repertoire of neuroendocrine-related polypeptides, including precursors of the neurohypophysial, tachykinin and insulin hormone families (Geenen et al., 1991). Previous studies from our laboratory have shown how the dual physiological role of the thymus may be, at least partially, explained by the two distinct types of molecular interactions engaged by thymic neuroendocrine-related polypeptides (Martens et al., 1996a). These precursors constitute a source of *cryptocrine signals* which are not secreted outside TEC, but targeted to TEC plasma membrane and able to bind to neuroendocrine-type receptors expressed by developing T-cells. The same precursors also constitute a source of *neuroendocrine self-antigens* which are presented by the thymic MHC system and which could, through this way, mediate central T-cell tolerance of their respective family.

Neurotensin (NT) is one of the first neuropeptides demonstrated by Sundler et al. (1978) to be expressed in the chicken thymic stromal cells. NT family mainly includes two bioactive peptides, namely NT (13 amino acids) and neuromedin N (6 amino acids). Both of these peptides

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PII S0165-5728(97)00052-0

originate from the same precursor, share similar pharmacological properties as well as the same tissue distribution. A number of central and peripheral actions of NT have been described, including hypothermic and analgesic effects in the central nervous system (CNS), stimulation of pancreatic exocrine secretion, vasodilatation and properties on the motility of the gastrointestinal tract (for complete reviews, see Aronin et al., 1983; Kitabgi and Nemeroff, 1992).

With regard to neurotensin-immune interactions, it has already been shown that interleukin-1α and tumor necrosis factor-α may regulate NT biosynthesis in chromaffin cells (Eskay and Eiden, 1992). Interleukins and insulin-like growth factors also increases NT production by the SH-SY5Y cell line derived from a human neuroblastoma (Loret et al., 1992). Immunocompetent cells were reported to express specific NT receptors (Evers et al., 1994). In T-lymphocytes, NT was shown to modulate adherence, chemotaxis and mitogenesis (Söder and Hellström, 1987; Garrido et al., 1992). In order to evaluate the hypothesis that thymic NT could also play a role in human T-cell differentiation, we investigated the intrathymic expression and presentation of NT-related peptides to immature T-lymphocytes.

2. Materials and methods

2.1. Cell cultures

Thymus fragments were obtained from children (6 months to two years) undergoing corrective cardiovascular surgery because of congenital cardiopathies. Finely scissed fragments were washed in Eagle's minimum essential medium containing D-valine supplemented with 2 mmol/l L-glutamine, 10 mmol/l HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum. The fragments were allowed to anchor in the same medium to T-75 flasks. At day 17, the adherent cells were detached using a Puck's modified solution containing trypsin and EDTA. Cells were counted and seeded at 6,000 cells/well in 12-well culture plates and used within 15 days. The technique of human TEC primary cultures has been described in detail elsewhere (Martens et al., 1996b).

2.2. Radioimmunoassay (RIA)

Intact NT and NT-ir fragments were detected using a specific RIA with an anti-NT antibody (Incstar) at the final dilution of 1:80,000. Synthetic NT (Sigma) was radiolabeled with ^{125}I using the lactoperoxydase method (Scarcériaux et al., 1994). The limit of detection was between 50 and 100 pg/ml. There was no significant cross-reaction with other NT-related peptides such as kinetensin and neuromedin N. A very weak partial but uncomplete cross-reaction was observed with xenopsin (ED50 > 10,000 ng/ml).

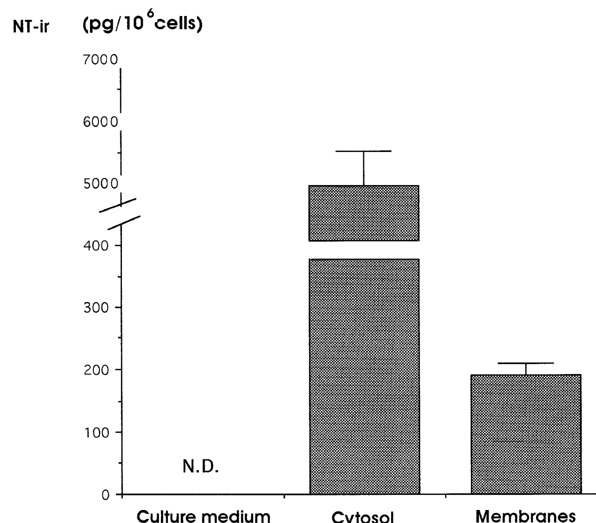

Fig. 1. Concentrations of NT-ir in culture medium, cytosol and membrane preparations from human cultured TEC. Values are expressed in pg/10^6 cells and are the mean ±SD of three independent experiments. N.D. means not detectable.

2.3. Preparation of human thymic stromal cell plasma membranes

Thymocytes were eliminated from human thymus by filtering on nylon gaze. Thymic fragments were mechanically homogenized in PBS 0.015 M (pH 7.3), then centrifuged for 10 min at 200 g. The supernatant was collected and supplemented with an equal volume of PBS 50 mM (pH 7.3). After centrifugation at 10,000 g for 10 min, the supernatant was collected and centrifuged again at 105,000 g for 60 min. The pellet was dissolved in buffered 0.5% NP-40, 5 mM sodium ortho-vanadate, 1 mM PMSF and 25 mM iodoacetamide and the final solution was filtered before further analyses.

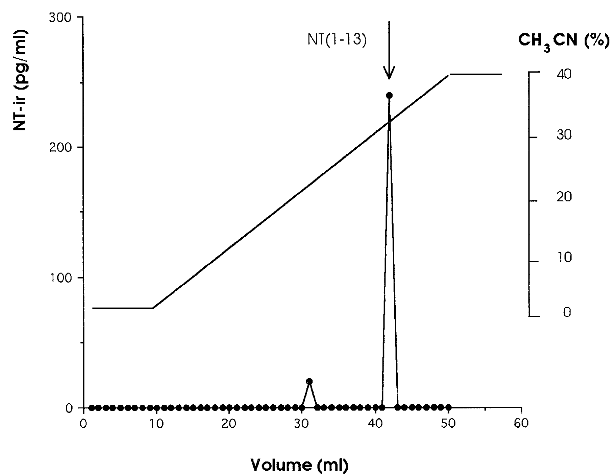

Fig. 2. HPLC profile of NT-like immunoreactivity from human cultured TEC. The cells were lyzed in distilled water and submitted to centrifugation. The supernatant from $6 \cdot 10^6$ cells/ml was injected into HPLC system. The arrow indicates the elution site of synthetic NT_{1-13}.

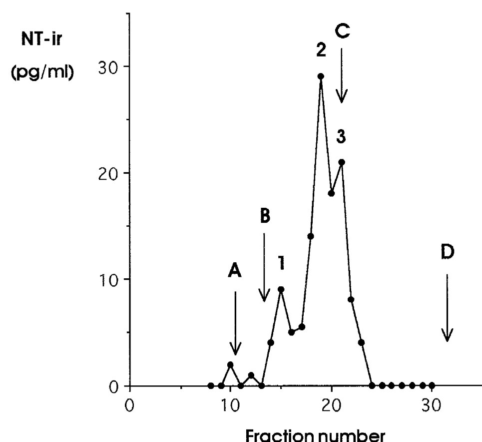

Fig. 3. FPLC separation profile of NT-ir extracted from thymic stromal cell plasma membranes. Three major peaks of NT-ir are evidenced corresponding to 40 kD (peak 1), 4 kD (peak 2) and to NT_{1-13} (peak 3). Arrows indicate the elution site of the standards used to calibrate the FPLC column. (A) dextran blue (void volume); (B) bovine serum albumin; (C) NT_{1-13}; (D) iodine (total volume).

2.4. Fast protein liquid chromatography (FPLC)

Thymic membrane extracts were submitted to FLPC using a Superose 12 column (30 × 1.0 cm, with a separation range between 1,000 and 300,000 kDa) (Pharmacia). The preparation was eluted at room temperature with a flow rate of 0.6 ml/min in ammonium acetate 0.3 M with 10% acetonitrile at pH 6.8. Fractions of 1.0 ml were collected, lyophilized and stored before NT RIA.

2.5. Affinity chromatography

Thymic membrane extracts were separated on an affinity column prepared with a mAb to MHC class I molecules, a IgG2A directed to the monomorphic determinant of human MHC (HLA) class I molecules (Rebaï and Malissen, 1983) according to the procedure previously described (Geenen et al., 1993a). Thymic HLA class I-related molecules were eluted from the column with buffered diethylamine at basic pH and the eluted fractions were submitted to high performance liquid chromatography (HPLC) analyses.

2.6. High performance liquid chromatography (HPLC)

Fractions eluted from the MHC-class I affinity column were separated by analytical reverse-phase HPLC on a 5μ-Bondapak C18 column (Waters Millipore) in 0.5% trifluoroacetic acid (TFA). Elution was carried out at room temperature in a linear gradient from 0 to 40% acetonitrile over a period of 40 min at a flow rate of 1.0 ml/min. Fractions of 1.0 ml were collected, lyophilized and resuspended in 100 μl ammonium bicarbonate 4 g/l, added with 4 g/l ovalbumin, 7 g/l EDTA, 0.5% Tween 20 and 50,000 IU trasylol before NT RIA.

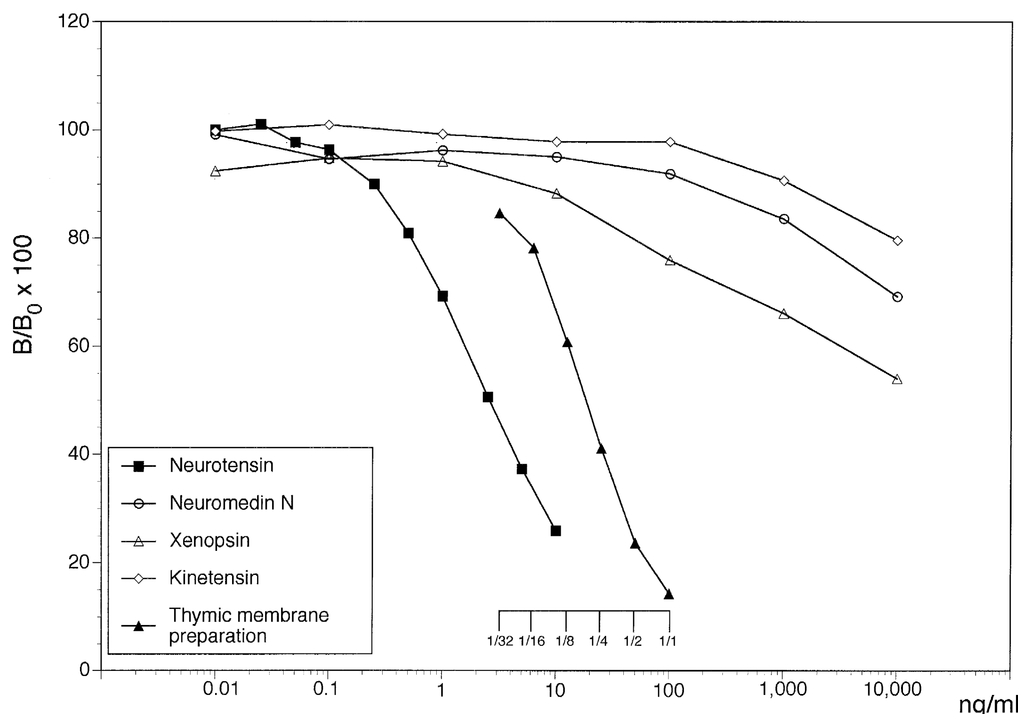

Fig. 4. Comparison of the displacement curves obtained with synthetic NT, with various analogues of NT and with a concentrated preparation of human thymic stromal cell membranes. The single data points are means of triplicate measurements.

3. Results

3.1. Identification of NT-like peptides in human TEC

Concentrations of NT-ir were measured using a specific RIA in TEC plasma membrane extracts, in TEC cytosol and in the incubation medium of primary TEC cultures (Fig. 1). NT-ir concentrations are 190 ± 20 pg and 4.95 ± 0.57 ng/10^6 cells (mean ± SD of three independent experiments) in TEC plasma membrane and cytosol, respectively. No NT-ir could be detected in the medium of human TEC primary cultures.

The reverse-phase HPLC profile of NT-ir extracted from human cultured TEC is shown in Fig. 2. The dominant peak of NT-ir is eluted with the same retention time than synthetic NT_{1-13}. A small peak of higher molecular weight NT-ir (±3% of total NT-like immunoreactivity) is also detected in fraction 31.

3.2. Membrane-bound NT-ir in human thymic membranes

The FPLC profile of NT-ir associated with proteins prepared from thymic stromal cell plasma membranes is presented in Fig. 3. Three peaks of NT-ir are evidenced corresponding, respectively, to MW around 40 kD (peak 1), 4 kD (peak 2) and to NT_{1-13} (peak 3). These peaks account respectively for 15, 48 and 37% of total NT-ir present in TEC plasma membrane preparations.

3.3. Immunoaffinity analysis

Fig. 4 shows the inhibition curve produced by synthetic NT, different NT-related peptides and by a concentrated preparation of human thymic stromal cell membranes. A close parallelism can be observed in the displacement of [^{125}I]-NT produced by synthetic NT and by the concentrated preparation of human thymic stromal cell membranes.

The amount of NT-ir retained on the anti-MHC class I column was about 13% (±36 pg/g wet weight) of the total NT-like immunoreactivity present in thymic stromal cell plasma membranes (±280–350 pg/g wet weight). Three peaks of NT-ir were evidenced by HPLC analysis of the fractions eluted from the affinity column (Fig. 5). One peak was eluted at the same position as synthetic NT_{1-13}, while the other peaks correspond to smaller fragments. In order to further characterize the latter, the HPLC column was calibrated with synthetic fragments of NT. None of the available standards (NT_{1-8}, NT_{1-11}, NT_{8-13} and NT_{9-13}) could help in further characterization of NT-ir eluted from the anti-MHC class I column. N-terminal standards are poorly recognized by the antibody used in the NT RIA and thus probably do not correspond to NT-ir detected by RIA in thymic extracts. On the other hand, C-terminal standards (NT_{8-13} and NT_{9-13}) both elute later than the thymic NT-ir-related fragments.

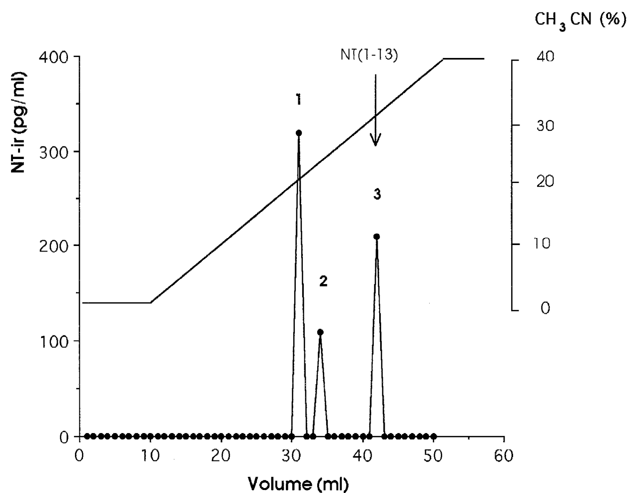

Fig. 5. HPLC analysis of NT-like immunoreactivity from the fractions retained on anti-MHC class I-affinity column and eluted with diethylamine in basic conditions (pH 10.5). Three peaks of NT-ir are detected. One of them (peak 3) coelutes with synthetic NT_{1-13}. The peaks 1 and 2 indicate the elution of NT-ir fragments smaller than NT_{1-13}.

4. Discussion

Even though various hormones have been identified in thymic extracts, there is no solid evidence arguing for the thymus as a true endocrine gland. Among thymic stromal cells, TEC represent the dominant component (±80% of thymic stromal cells). The pattern of secretion in TEC had already been shown to differ from the classical (neuro)endocrine secretory pathways (Nabarra and Andrianarison, 1987). With regard to thymic oxytocin (OT), it was demonstrated that the peptide is not located in classical secretory granules, but is diffuse within TEC cytosol, in large clear vacuoles and around perimembranar space (Wiemann and Ehret, 1993). From these data it was concluded that OT is not secreted by TEC and we have further shown that OT is targeted to TEC plasma membrane. In addition, several types of experiments have established that thymic OT behaves like the self-antigen of the neurohypophysial hormone family (Geenen et al., 1991, 1993a; Robert et al., 1992; Martens et al., 1996b).

The presence of NT-related immunoreactivity in the chicken thymus was first reported by Sundler et al. (1978). Using immunocytochemistry, RIA and chromatographic analyses, this team showed that unidentified thymic stromal cells express NT-ir appearing to have a larger molecular weight than native NT_{1-13}. Our study confirms the presence of the whole NT molecule in human cultured TEC. As it was shown for thymic OT, the present work demonstrates that NT-ir sequences are associated to MHC class I molecules. The hypothesis of an intrathymic presentation of NT-ir to pre-T cells is supported by the following observations:

(1) Though intact NT_{1-13} is produced and expressed within TEC (Fig. 2), no detectable secretion of the peptide was observed in the extracellular medium. This unexpected behavior was also observed for OT-ir and IGF-II-ir expressed by the same thymic cell type (Geenen et al., 1993a,b).

(2) FPLC analysis of membrane enriched-preparations of thymic cells revealed a peak of NT-related immunoreactivity associated to a 40–50 kD protein. This molecular weight is compatible with the size of a MHC class I heavy chain (45 kD). Although the peak 2 of NT-ir has not been sequenced, it is noteworthy that a 4 kD NT-related peptide could be obtained from minor cleavage site in the NT precursor, located upstream of the neuromedin N sequence (Carraway et al., 1992).

(3) Using an affinity column prepared with an anti-MHC class I mAb, NT-ir can be detected within the fractions retained on the column and eluted with diethylamine in basic conditions. At the light of the control experiments with NT-derived fragments, it can be suggested that the MHC-associated NT-like peptides could be either intact NT_{1-13} or NT C-terminal smaller fragments which are recognized by the anti-NT antibody used in this study. Interestingly, such a pattern has been described for other peptides identified in the immune system. Jessop et al. (1994) showed that at least two isoforms of ACTH and β-endorphin are expressed by thymic cells, while thymic neuropeptide Y appeared as two distinct peaks after separation by reverse-phase HPLC (Jessop et al., 1992).

Altogether, these observations strongly suggest that a significant part of thymic NT is presented by thymic MHC class I-related molecules to differentiating T-cells. The question of the peptide length required for anchoring to the pocket of MHC class I molecules has been addressed in many papers. Though 8–9 amino-acid sequences are able to fit the best affinity parameters for MHC binding, several authors have reported the association of larger sequences (10–18 amino acids) with MHC (Chen et al., 1994; Collins et al., 1994). These peptides use a specific anchorage pattern providing affinity constants quite similar to those obtained with nonamers (Urban et al., 1994). Therefore, the association of intact NT1–13 with MHC class I seems very plausible. Moreover, NT exhibits two characteristics playing in favor of a binding to MHC. First, NT is a linear peptide with minimal physical constraint for a non-covalent binding to MHC. Second, the C-terminal sequence of NT includes tyrosine, isoleucine and leucine residues. Each of the residues can be used in the anchorage to many MHC alleles (Rammensee et al., 1993). In contrast to cyclic OT or IGF-II, there is no particular structural constraint for the presentation of NT by a large number of MHC class I alleles. Given its biochemical characteristics, it seems logical to postulate that NT and NT-derived C-terminal fragments could behave as natural ligands for a majority of MHC class I alleles. In addition, this hypothesis is in agreement with the high degree of conservation of the NT-related C-terminal region throughout evolution (Carraway et al., 1982).

The thymic T-cell education to the NT family during fetal life is also supported by the finding that, in newborn children, the concentrations of circulating NT are 8–10 fold higher than in adults. In addition, the main form of plasma NT-ir in these children correspond to molecules of 40 kD which are not present in adult blood (Bozzola, personal communication).

Acknowledgements

This work was supported by the Belgian NFSR, by the Association contre le Cancer (Belgium) and by the grant ERB-BMH1-CT 93-5193 from the European Union to Y.V. Unité INSERM 339, Hôpital Saint-Antoine, F-75571 Paris, France.

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