Epidermal Growth Factor Promotes a Neural Phenotype in Thymic Epithelial Cells and Enhances Neuropoietic Cytokine Expression

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Abstract. Neural crest-derived cells populate the thymus, and their coexistence with epithelial cells is required for proper organ development and T cell education function. We show here that epidermal growth factor (EGF), a major epithelial cell growth-enhancing agent, has a morphogenetic action to promote the expression of a neuronal phenotype (e.g., neurofilament expression) in cultured thymic epithelial cells that are characterized by a cytokeratin-positive epithelial cell background. The proliferation of such neurodifferentiated cells is also enhanced by EGF. Furthermore, the growth factor enhances cells that express the genes encoding the preprotachykinin A-generated neuropeptides and the bipotential neuropoietic and lymphopoietic cytokines ciliary neurotrophic factor and interleukin-6. These cytokines also enhance the neuronal phenotype of thymic epithelial cells. Therefore, EGF appears to be a composite autocrine/paracrine neuromodulator in thymic stroma. This suggests that EGF may regulate thymus-dependent immune functions by promoting neuronal gene expression in neural crest-derived cells.

MULTIPLE cell interactions control developmental cell fate and morphogenesis. The morphogenetic process of the thymus is an example in which the variety of phenotypes resulting from T lymphoid cell developmental pathways and the acquisition of either the ability to react to external antigens or the tolerance against self-antigens arise through the action of instructive and/or selective differentiation signals generated by the thymic stroma in close association with precursor and differentiating lymphoid cells (8, 47, 51). A striking aspect of the thymic stroma is the coexistence of cell types arising from distinct cell lineages represented mainly by the endoderm of the third pharyngeal pouch, the ectoderm of the third branchial cleft and cells from the corresponding region of the neural crest (35, 36). The interaction of neuroectoderm-derived cells with other epithelial cells is necessary for both the organ morphogenic and the T cell differentiation processes. Indeed, ablation of the cephalic neural crest in chicken embryos results in thymic aplasia/hypoplasia (4, 7). The further fate and specific functions of neural crest-derived cells within the thymic stroma remain unclear, although the presence of neural crest-derived cells in thymic medulla and the expression of a number of neuronal markers and neuropeptides in both thymic epithelium in vivo (14, 18, 20, 42) and thymic stromal cell cultures (50) have been described. Cytokeratin-harboring thymic nurse epithelial cells also express most of these neuron and neuroendocrine markers and neuropeptides (19).

The coexistence of epithelial and neural cells within the thymic stroma raises the question as to the nature of the regulatory signals that determine the development and survival of the neuronal component. EGF is a likely candidate as a soluble regulatory signal. Indeed, in addition to being a potent growth factor for several epithelial cell types (10), EGF and its receptor are expressed in the central nervous system (CNS)[1] (15, 21) and the growth factor promotes both neuron survival and mitogenesis of neuronal progenitors derived from neonatal rat retina and adult mouse striatum (1, 41, 45, 59). Furthermore, EGF receptor ligands (EGF and TGFα) are also expressed by thymic tissue in vivo and by human thymic epithelial cell

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1. *Abbreviations used in this paper*: BrdU, bromodeoxyuridine; CNTF, ciliary neurotrophic factor; EGF, epidermal growth factor; IL-6, interleukin-6; NF, neurofilament; PPT-A, preprotachykinin-A; RT-PCR, reverse transcription-polymerase chain reaction; SYN, synapsin-1; TC, thymic stromal cell; TH, tyrosine hydroxylase.

cultures, in which these factors modulate the production of several cytokines (including interleukin-6 [IL-6]) (25, 32). The ability of EGF to enhance IL-6 production is of particular interest. IL-6 belongs to the neuropoietic cytokine family that influences the survival, proliferation, and differentiation of neurons (12, 24). We have previously shown that IL-6 is able to enhance the neural phenotype of thymic stromal cells (50). The aim of this work is therefore to study the role of EGF in regulating the neural cell population in thymic stroma and the neurotrophic microenvironment sustained by these cells.

Material and Methods

Thymic Stromal Cell Cultures

Several murine thymic stromal cell (TC) cultures were independently established from 4–5-wk-old C57BL/6 mice as previously described (38, 50). Human thymic stromal cell (HTC) cultures were also independently established from thymuses of children undergoing cardiac surgery. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 0.1 mg/ml sodium pyruvate and antibiotics (Hyclone Europe Ltd., Cramlington, UK). All experiments reported were performed with TC cultures from the 3rd to 40th passage.

Flow Cytometry and Cell Cycle Analysis

Cell cycle analysis was performed by fixing 1 x 10^6 control or 48 h EGF-treated cells in 70% cold ethanol for 30 min at 4°C and adding, after two washes in cold PBS, 0.5 mg/ml RNAse (Sigma Chemicals Co., St. Louis, MO), and 40 μg/ml of propidium iodide (Sigma). Cells were then kept in the dark at 4°C for 30 min and immediately analyzed by flow cytometry. The propidium iodide fluorescence emission, due to DNA content of individual cells, was filtered through a 585/42-nm band pass filter and measured on a linear scale using a FACScan cytometer (Becton Dickinson, Mountain View, CA). Cell debris and doublets were excluded from analysis by appropriate gating using physical parameters.

Immunofluorescence and Immunoperoxidase Staining

Immunofluorescence staining was performed using cells grown on slide culture chambers, in the absence or presence of 10 ng/ml of EGF (Collaborative Res. Inc., Bedford, MA), 0.5 ng/ml recombinant murine IL-6 (British Biotechnology, Oxford, UK), or 10 ng/ml recombinant rat ciliary neurotrophic factor (CNTF; Genzyme, Cambridge, MA). After washing with PBS, cells were immediately fixed with ice-cold absolute ethanol for 5 min and incubated with various primary polyclonal or monoclonal antibodies for 1 h at room temperature, washed with PBS, and then incubated with Fluorescein (FITC)-conjugated goat anti–rabbit or anti–mouse (Fab') IgG (Sigma) for 45 min at room temperature. For EGF receptor staining, cells were preincubated for 2 h in medium without FCS and then fixed with 2% buffered paraformaldehyde for 10 min at room temperature. Primary antibodies used included polyclonal rabbit anti-EGF and anti-EGF receptor (Oncogene Science, Manhasset, NY), monoclonal anti-tyrosine hydroxylase (clone 2/40/15) and anti-neurofilaments (NF) 200 kD (clone NE14) (both from Boehringer Mannheim GmbH, Mannheim, Germany), anti-NF 160 kD (Amersham Corp., Buckingamshire, UK), pan-cytokeratin monoclonal antibody (clone LU5, Boehringer Mannheim GmbH), rabbit polyclonal anti-synapsin I antiserum (a gift from P. De Camilli, Yale University School of Medicine, New Haven, CT), and monoclonal anti-CNTF (Boehringer).

Double-immunofluorescence staining was performed by labeling cells with anti-cytokeratin monoclonal antibody followed by fluorescein-conjugated goat anti–mouse IgG and then with anti-NF 200 kD rabbit polyclonal antibody (Sigma) followed by rhodamine-conjugated anti-rabbit IgG (ICN ImmunoBiologicals, Lisle, IL). Fluorescent images were analyzed with a fluorescence microscope (DMRB; Leitz, Wetzlar, Germany) or a confocal laser scanning microscope (TCS 4D; Leica, Inc., Deerfield, IL). In the latter case, the green (FITC) and the red (rhodamine) fluorescences were independently recorded and elaborated through a 3-D function (Extended Focus or Shadow Forming Projection). The 3-D–obtained images were then independently observed or electronically superimposed to simultaneously demonstrate the double staining.

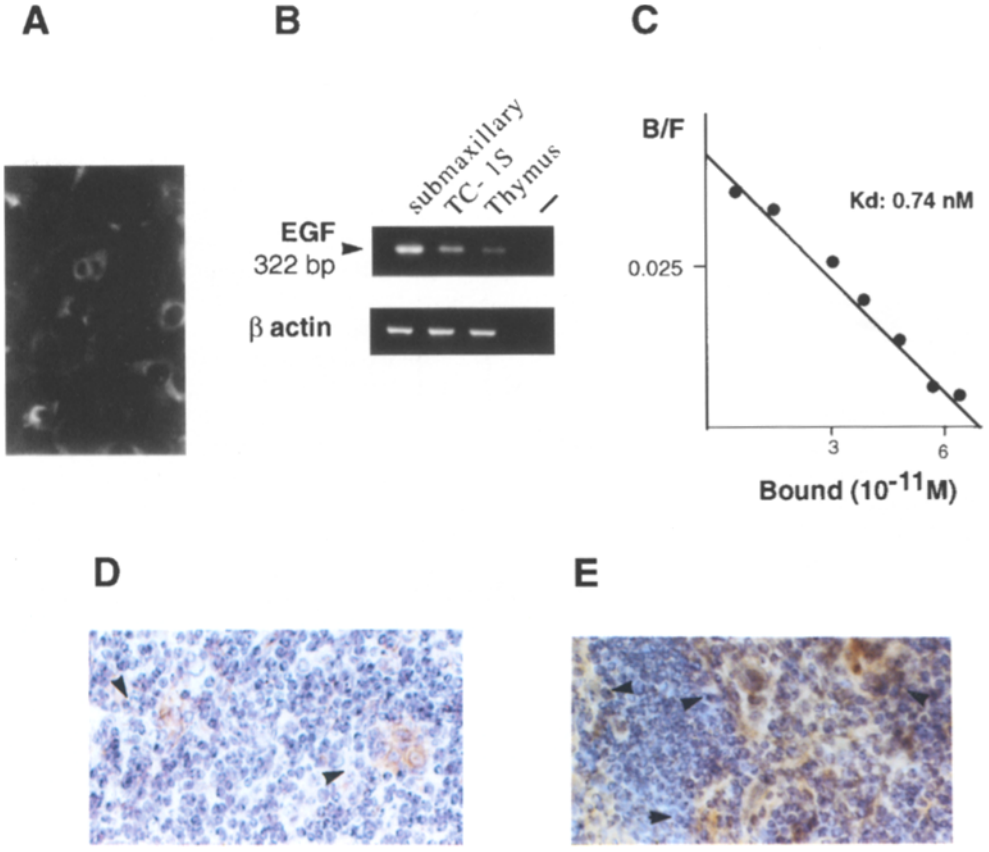

Figure 1. Expression of EGF and EGF receptor in TC-1S cells and thymic tissue. (*A*) Fluorescence photomicrograph of cells immunoreactive for EGF. (*B*) RNA isolated from mouse submaxillary glands or thymus or TC-1S cells was processed for RT-PCR using either EGF or β-actin primers as described. Results are shown as ethidium bromide staining of amplified products. RT-PCR reaction of the last lane does not contain RNA. (*C*) Scatchard plot of I^{125}-EGF binding to TC-1S cells. (*D* and *E*) Immunoperoxidase staining of murine thymic sections showing immunoreactive EGF receptors (*D*) and EGF (*E*) (*arrowheads*).

Bromodeoxyuridine (BrdU) labeling was performed using an Amersham Cell Proliferation kit (Amersham Corp.) according to the manufacturer's instructions. Briefly, the cells were incubated for 1 h at 37°C with 5′-bromo-2′-deoxyuridine and 5′-fluoro-2′-deoxyuridine and then fixed in 90% ethanol/5% acetic acid, rehydrated with PBS and stained with anti-BrdU antibody and then revealed with peroxidase–anti-mouse IgG2a and diaminobenzidine intensified with cobalt chloride and nickel chloride. NF staining was demonstrated by immunoperoxidase staining using anti-NF-200 kD antibody and a streptavidin-biotin system (Histostain SP kit; Zymed). Cells were counterstained with hematoxylin. Substance P was detected by immunoperoxidase staining as described above. Cryostat tissue sections from mouse thymus were stained using anti-EGF or anti-EGF receptor antibodies by immunoperoxidase staining.

Reverse Transcription Polymerase Chain Reaction and Northern Blot Analysis

Reverse transcription polymerase chain reaction (RT-PCR) was performed by a first step of reverse transcription from DNaseI-treated RNA isolated from TC-1S cells and brain for 15 min at 42°C, followed by 5 min at 99°C and then 5 min at 5°C (GeneAmp RNA PCR Kit in a 20-μl vol containing 5 mM $MgCl_2$, 2 μl PCR buffer, 1 mM each deoxynucleotide, 1 U RNase inhibitor, 2.5 U Reverse Transcriptase; Perkin-Elmer Cetus Corp., Norwalk, CT). Amplification of synthesized cDNA was then performed using specific sense and antisense primers described below and reagents of the GeneAmp RNA PCR Kit in a 100-μl reaction volume. Each sample was overlaid with 50 μl of mineral oil (Sigma) and incubated in a DNA thermal cycler controller. PCR products were analyzed by 2% agarose gel electrophoresis followed by either ethidium bromide staining or Southern blotting and hybridization using specific oligomers internal to the amplified sequences. β-actin amplification using 5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-3′ as 5′ and 3′ primers, served as an internal control for both reverse transcription and the PCR. The 5′ and 3′ CNTF primers were 5′-CCAGTGGCAAGCACTGATC-3′ and 5′-CCCATAATGGCTCTCAAGTGC-3′, respectively. The 5′ and 3′ CNTF-Rα primers were 5′-GCTGTCACATTCGGTACATGC-3′ and 5′-AGCCACACTCCAGTCACTCCA-3′, respectively. The 5′ and 3′ IL-6-Rα primers were 5′-TTCATGATGCCTTGCGAGGA-3′ and 5′-TCATAAGGGCTCTGTGCGTC-3′, respectively. The 5′ and 3′ PPT-A primers were 5′-AGCGACCAGATCAAGGAGG-3′ and 5′-GCACTCCTTTCATAAGCCAC-3′, respectively. The 5′ and 3′ EGF primers were 5′-TGTGTTATTGGCTATTCTGG-3′ and 5′-TCTTGGGGTCTTGGTGTTTCT-3′, respectively. Amplified fragments were hybridized with the following specific internal sequences: 5′-TTGAGGCTGATACATCGACTGAACATCAAT-3′ (for CNTF-Rα); 5′-ATGTGGGCAGGGACATGGACGAGGATTCTT-3′ (for IL-6-Rα). Bands were quantitated by scanning densitometry using a GS-670 scanning densitometer (Bio-Rad Labs., Richmond, CA) and employing Molecular Analyst PC™ software.

Results

EGF and EGF Receptor Expression in Cultured Thymic Epithelial Cells and in Thymic Tissue

We have established and previously characterized several thymic epithelial cell lines (TC) that are mostly represented by cytokeratin-positive epithelial cells that are able to support thymocyte differentiation (38, 50). These lines also contain a minor NGF-sensitive neural cell population, which express synapsin-I and neuron-specific cytoskeletal neurofilaments (50).

The existence of an autocrine/paracrine loop involving EGF in TC cultures was suggested by immunofluorescent staining of intracytoplasmic EGF in ~25% of the cells (Fig. 1 *A*) and by the presence of EGF mRNA (Fig. 1 *B*). Furthermore, cell surface EGF receptors were observed in ~50% of the cells, as revealed by anti-EGF receptor immunofluorescence staining (not shown). These receptors bound ^{125}I-EGF with a K_d of 0.74 ± 0.1 nM and had a concentration of 35,000 ± 3,000 sites/cell (Fig. 1 *C*). The production of EGF was confirmed in thymic tissue ex vivo which expresses EGF mRNA (Fig. 1 *B*). Immunostaining for both EGF receptor (Fig. 1 *D*) and EGF (Fig. 1 *E*) was observed in epithelial cells of thymic medulla and cortex from ex vivo tissues.

EGF Enhances the Neuronal Phenotype of Thymic Epithelial Cells

The addition of EGF to several murine or human TC cultures was able to enhance their neuronal phenotype by

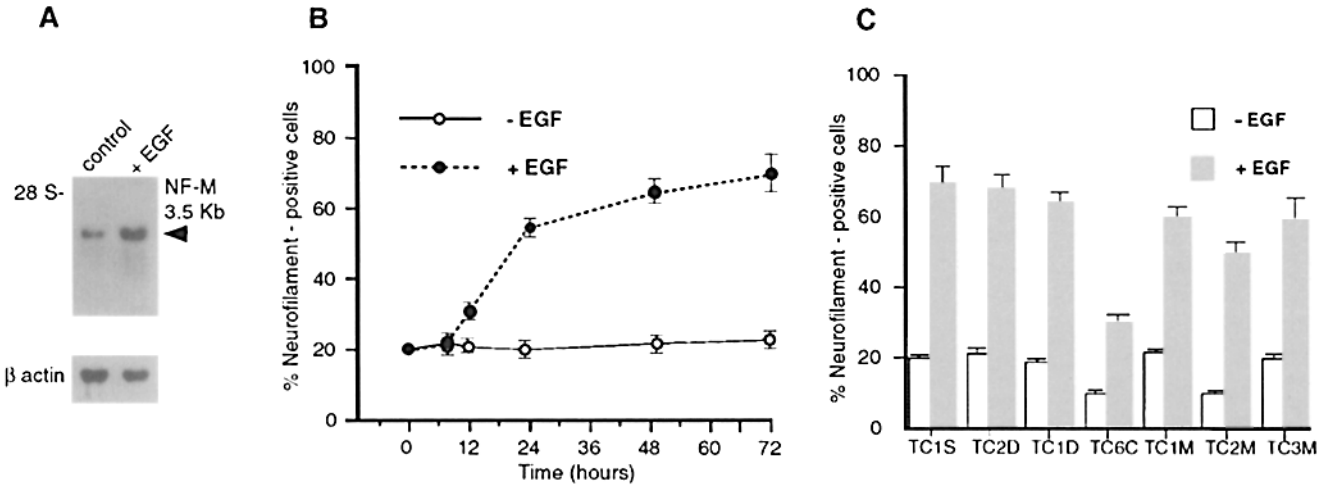

Figure 2. (*A*) Enhancement of NF mRNA levels by EGF. Total RNA was isolated from TC-1S cells, untreated or treated for 48 h with EGF and processed for Northern blot analysis as previously described (12) using a NF-160 kD (*NF-M*)–specific ^{32}P-labeled probe (pNf36; American Type Culture Collection) (*top*) and, after stripping, a β-actin probe (*bottom*). 28S rRNA and the 3.5-kb NF-M mRNA are indicated. (*B*) Time course of EGF activity on the percentage of neurofilament-positive cells relative to the total TC-1S cell population. (*C*) Percentage of neurofilament-positive cells relative to the total cell population in the absence of EGF (− *EGF*) or 48 h after the addition of the growth factor (+ *EGF*) in several murine TC cultures.

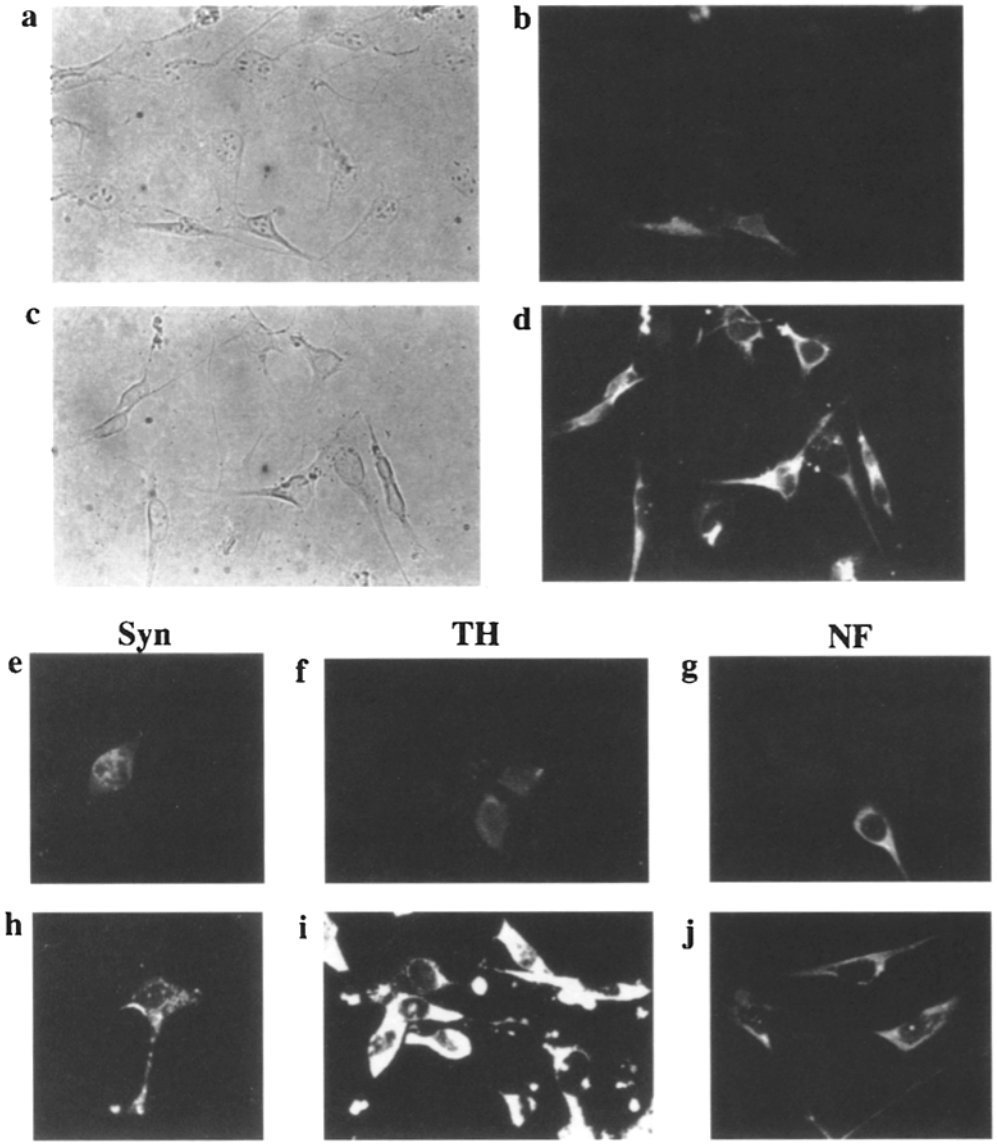

Figure 3. Morphology and phenotype of TC-1S cells untreated (*a*, *b*, *e*, and *f*) or treated with EGF for 48 h (*c*, *d*, *h*, and *i*). Phase contrast (*a* and *c*) and fluorescence photomicrographs of cells immunoreactive for neurofilaments (*b* and *d*), synapsin I (*e* and *h*) and tyrosine hydroxylase (*f* and *i*). Fluorescence micrographs of human TC cultures immunoreactive for neurofilaments in the absence (*g*) or in the presence of EGF treatment (*j*). Immunoreactive cells display long and fine processes.

significantly increasing the levels of NF mRNA and both 160- and 200-kD NF immunofluorescent staining intensity (Figs. 2 *A* and 3, *b* and *d*).

The percentage of NF^+ cells was also increased by EGF in several cultures from murine or human thymuses (Fig. 2, *B* and *C* and Table I). NF^+cells also showed significant elongation of neurite-like processes after EGF treatment (Fig. 3). Most of the TC-1S cells displayed a similar cytokeratin expression under both basal conditions and following EGF-treatment. This suggests an overlapping of cytokeratin-positive and NF^+ cells. NF expression has been recently reported to be confined to cytokeratin-positive epithelial cells in thymic neoplasms derived from cortical epithelium of the thymus (37). Therefore, to study the relationships between the EGF-induced increase in NF expression and cytokeratin$^+$-epithelial cells, we double-stained TC-1S cells with anti-cytokeratin and anti-NF antibodies. Figs. 4, *b* and *c* and 5, *a* and *b* show that NF expression was only observed in cytokeratin-positive cells. It is also evident from Figs. 4 and 5 that, while cytokeratin maintains the same paranuclear localization in both untreated and EGF-treated cells, the EGF-induced increase of NF expression is mainly evident in the cell periphery, along the cytoplasmic processes. EGF increased NF expression in ~70% of the cells which also coexpressed cytokeratin (Figs. 4, *e* and *f* and 5, *c* and *d*), while we have been unable to detect NF in cytokeratin-negative cells. This suggests an EGF-mediated enhancement of the neural phenotype within the epithelial cell population itself.

Table I. Percent (Mean ± SD) of NF or TH-positive or SYN-positive Cells in Human TC Cultures before (Control) and after EGF Treatment

Human cell line	Control			EGF		
	NF	TH	SYN	NF	TH	SYN
HTC-3	22 ± 3	10 ± 3	5 ± 1	60 ± 7	40 ± 8	10 ± 2
HTC-14	20 ± 2	10 ± 2	8 ± 3	65 ± 9	35 ± 5	20 ± 3
HTC-16	21 ± 2.2	8 ± 1.5	5 ± 1.5	57 ± 5	20 ± 4	25 ± 4
HTC-19	16 ± 1.5	5 ± 2	4 ± 1	48 ± 6.2	20 ± 4	10 ± 3

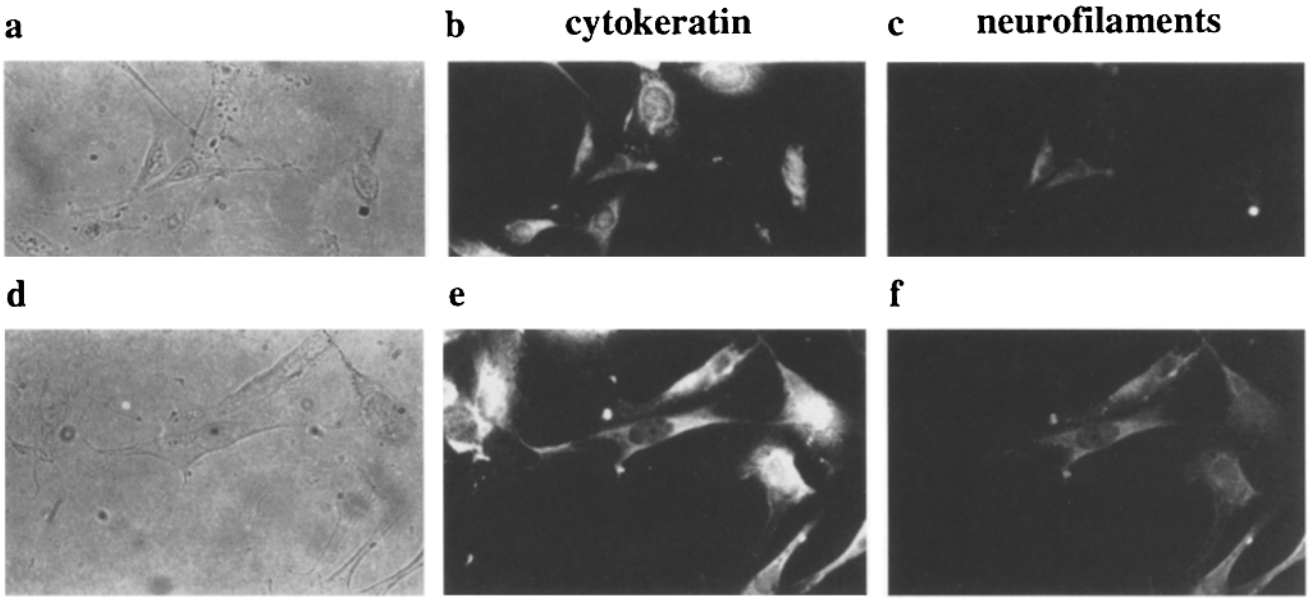

Figure 4. Phase contrast micrographs (*a* and *d*) and double immunofluorescence staining (*b*, *c*, *e*, and *f*) of TC-1S cells untreated (*a–c*) or treated for 48 h with EGF (*d–f*). TC-1S cells were stained with anti-cytokeratin (*b* and *e*) and anti-neurofilament (*c* and *f*) antibodies.

EGF also enhanced the expression of other markers specific for neuronal and/or neuroendocrine cells including synapsin 1 and tyrosine hydroxylase (TH) (Fig. 3, *e*, *f*, *h*, and *i*). The percentage of TH^+ cells increased from 9 ± 2% to 35 ± 4% and that of $synapsin^+$ (Syn) cells from 7 ± 2% up to 30 ± 6%, 48 h after the addition of EGF. Similar results were observed in human thymic epithelial cells (Fig. 3, *g* and *j* and Table I).

To study whether the EGF-induced increase of neural cell number in TC cultures was due to an enhanced proliferation rate of NF^+ cells, we analyzed the effect of EGF on TC-1S cell growth. Cell cycle analysis showed that an increased percentage of cells were in S and G2/M phases 24 h after EGF treatment compared to untreated TC-1S (Fig. 6 *A*). Moreover, labeling TC-1S cells with BrdU and double staining with anti-NF and anti-BrdU antibodies

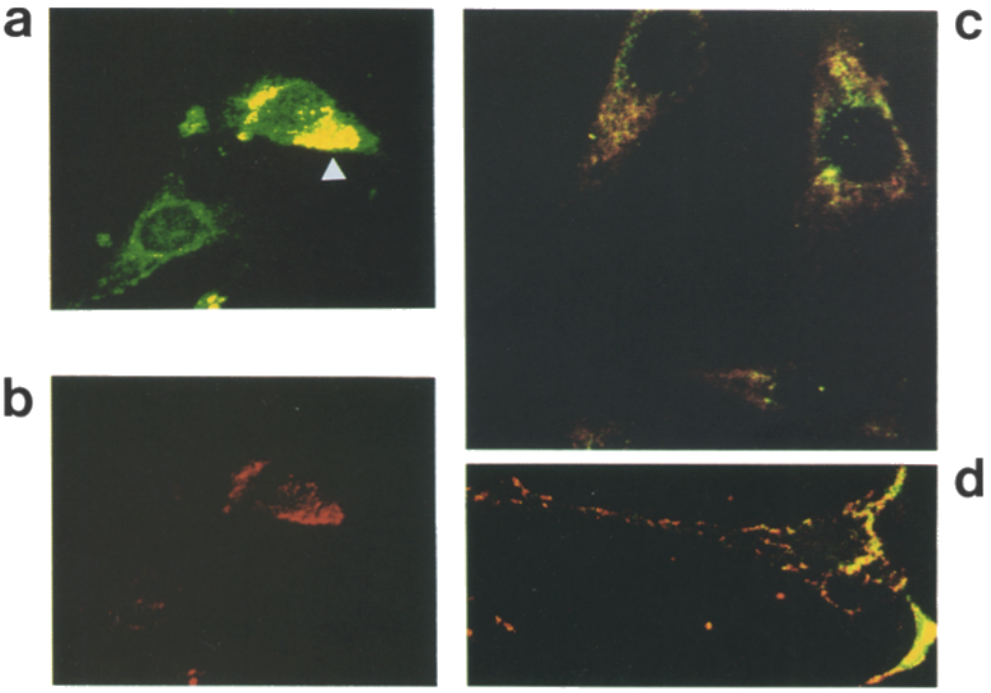

Figure 5. Double immunofluorescence staining of TC-1S cells untreated (*a* and *b*) or treated for 48 h with EGF (*c* and *d*). TC-1S cells were stained with anti-cytokeratin (green fluorescence) and anti-NF (red fluorescence) antibodies and analyzed with a confocal laser scanning microscope. *a* shows the double fluorescence photomicrograph of cytokeratin-positive TC-1S cells, one of which also coexpresses neurofilaments (*arrow*). The arrow indicates the region of the cell where cytokeratin and neurofilaments coexist and are overlapping. *b* shows the NF immunoreactivity alone of the same cell indicated by the arrow in *a*. (*c* and *d*) Simultaneous representation of double immunofluorescence staining showing cells which coexpress cytokeratin and neurofilaments that are distributed in distinct (separated green and red signals) or overlapping (*yellow*) regions. In *c*, cytokeratin appears to be localized in the perinuclear area while neurofilaments are mostly expressed along cytoplasmic processes. *d* indicates a cell displaying a neurite-like process harboring neurofilament immunoreactivity and both neurofilament and cytokeratin coexpression in the cell body.

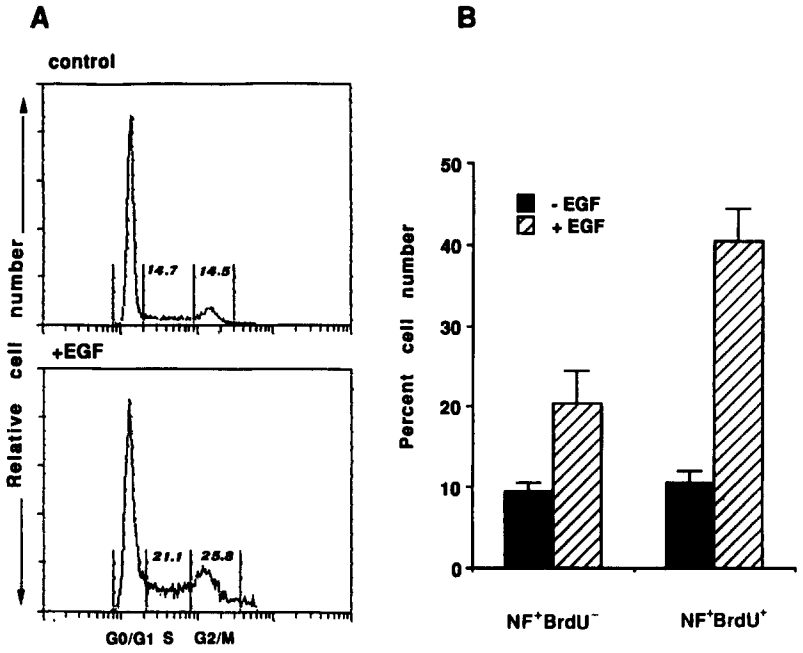

Figure 6. Effect of EGF on the growth rate of TC-1S cells. (*A*) Flow cytometry cell cycle analysis of TC-1S cells untreated (*control*) or treated with EGF (*+EGF*) for 24 h. Numbers indicate the percentage of cells in the G_0/G_1, S or G_2/M phases of the cell cycle. (*B*) BrdU-labeled TC-1S cells either untreated (*−EGF*) or treated (*+EGF*) for 24 h with EGF. Results are expressed as percentage of cells labeled with BrdU and/or anti-NF antibody.

showed that EGF addition resulted in a significant increase of the percentage of NF^+ cells incorporating BrdU (Fig. 6 *B*). This suggests that EGF enhances the proliferation rate of NF^+ cells.

Effect of EGF on Neuropeptide Production by Thymic Epithelial Cells

To study whether the EGF-induced neurotypic response in thymic stroma also included the ability to influence the production of specific neurotransmitters, we studied the expression of the preprotachykinin A (PPT-A) gene that encodes substance P and Neurokinin A. Scattered cells in thymic stroma have in fact been previously shown to express both neuropeptides in vivo (14). We show here that TC-1S cells express the β and γ transcripts of the PPT-A gene (Fig. 7 *A*). The EGF-induced neurotypic response in thymic stroma included an increase of cells able to produce these PPT-A–derived neurotransmitters. EGF induced a twofold and a sixfold increase of PPT-A mRNA levels (after 4 and 24 h of treatment, respectively, as evaluated by densitometric scanning) (Fig. 7 *B*). Furthermore, cells expressing substance P were barely detectable before EGF addition, following which substance P-expressing cells accounted for 25 ± 5% of the total population (Fig. 7 *C*).

Effect of EGF on Neuropoietic Cytokine Production

Several neurotrophic and neuropoietic factors are able to enhance the neuronal phenotype and neuropeptide expression. To study whether the EGF-induced neurotypic response also includes specific neuropoietic factors, we studied the ability of EGF to influence the expression of members of the neuropoietic cytokine family. Indeed, the previously reported ability of EGF to upregulate IL-6 expression in human thymic epithelial cells (32), makes members of this cytokine family (e.g., IL-6 and CNTF) likely candidate targets of EGF regulation. Such cytokines share signal transduction coreceptor subunits and influence both the immune and nervous systems since they are able to regulate both hemo-lymphopoiesis and survival and differentiation of neurons (12, 24). We first studied the expression of IL-6 and CNTF in TC-1S cells in the absence of EGF treatment. Whereas IL-6 mRNA was observed by Northern blot analysis (Fig. 8 *D*), CNTF transcripts were detectable only by increasing the sensitivity of the assay by the use of RT-PCR (Fig. 8 *A*). Similarly, only very low expression of CNTF protein was observed (2–4% of CNTF-immunoreactive cells). The presence of CNTF in thymic stromal cells, albeit at a low level, was striking, given the predominant expression of this cytokine in the astrocytes and Schwann cells of the peripheral and central nervous system (16, 43, 56). By using RT-PCR, we observed that EGF significantly increased CNTF mRNA levels by threefold as early as 4 h after treatment with a further increase (by 15-fold) after 24 h (Fig. 8 *A* and not shown). The EGF-induced enhancement of CNTF mRNA expression was also confirmed by Northern blot analysis using poly(A)$^+$-selected cellular RNA, which showed significant expression of CNTF mRNA 24 h after EGF treatment (a 30-fold increase of CNTF mRNA levels compared to untreated cells) (Fig. 8 *B*). The percentage of CNTF-immunoreactive TC-1S cells was also increased by EGF (22 + 3% of $CNTF^+$ cells 48 h after EGF-treatment) (Fig. 8 *C* and not shown). IL-6 gene expression was enhanced by EGF in murine TC-1S cells: IL-6 mRNA levels were increased by 15-fold and by 10-fold 1 and 24 h, respectively, after EGF treatment, compared to untreated cells (Fig. 8 *D* and not shown).

CNTF and IL-6 Enhance the Neuronal Phenotype of TC-1S Cells

The enhanced expression of CNTF and IL-6 by EGF suggests that these cytokines may contribute to the neurotypic effect of this growth factor. IL-6 or CNTF activities have been previously reported to require the presence of

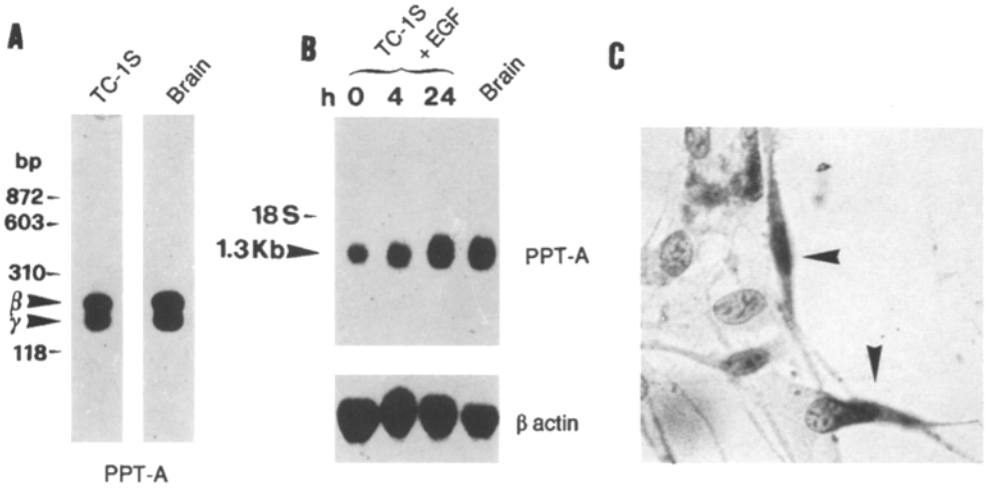

Figure 7. Enhancement of PPT-A gene expression by EGF in TC-1S cells. (*A*) Southern hybridization of amplified products obtained by RT-PCR of PPT-A gene transcripts in RNA of TC-1S and mouse brain. Arrowheads indicate β and γ PPT-A transcripts. (*B*) Northern blot analysis of PPT-A mRNA in TC-1S cells untreated and 4 and 24 h after EGF addition. The 5′-TAATCCAAAGAACT-GCTGAG-GCTTGGGTCT-3′oligonucleotide (corresponding to the substance-P encoding region) was used as hybridization probe in both *A* and *B*. (*C*) Immunoperoxidase staining of TC-1S cells treated for 48 h with EGF describing cells with elongated processes showing immunoreactive substance P (*arrowheads*).

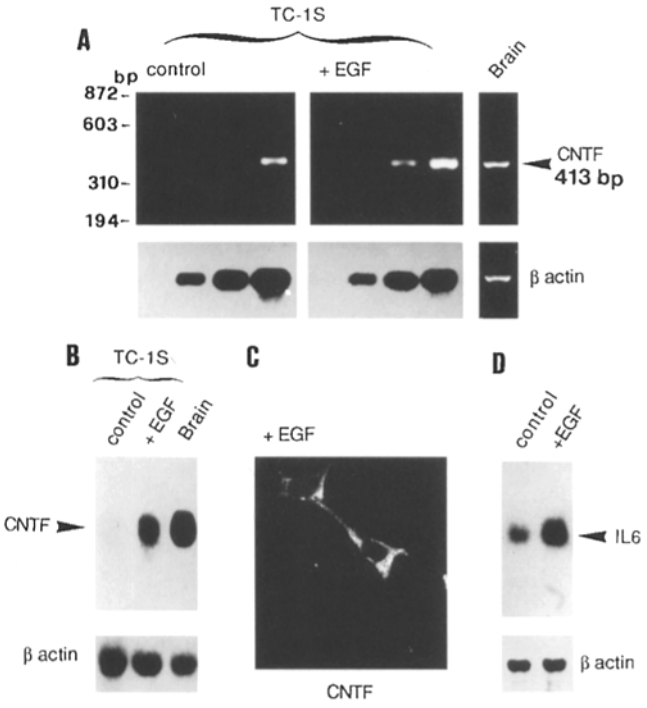

Figure 8. Expression and modulation of CNTF and IL-6 in TC-1S cells. (*A*) RNA isolated from TC-1S cells cultured in the absence (*control*) or in the presence of EGF for 24 h (+*EGF*) or from mouse brain was processed for RT-PCR using CNTF primers as described. Samples of the PCR reactions were taken at multiple points throughout the CNTF amplification (21, 24, 30, and 30 cycles), allowing the analysis of the product during the exponential phase of DNA amplification. Brain CNTF was amplified for 30 cycles. Results are shown as ethidium bromide staining of CNTF amplified products (*upper panel*) or Southern blot hybridization of β-actin (*lower panel*). (*B and D*) poly(A)$^+$ RNA isolated from untreated (*control*) or 24 h EGF-treated (+*EGF*) TC-1S cells or total RNA from mouse brain was processed for Northern blot analysis using a CNTF cDNA probe obtained from rat brain by RT-PCR (*B*) and murine IL-6 cDNA (56) (*D*) and, after stripping, a β-actin probe (*lower panels*). (*C*) Fluorescence microphotograph of TC-1S cells immunoreactive for CNTF 24 h after EGF treatment.

at least two receptor subunits, one of which is either the specific IL-6-Rα or CNTF-Rα subunit and the other a shared gp130 signal transduction moiety (12, 24). We detected transcripts encoding both the α subunit of the IL-6 and CNTF receptors in TC-1S cells (Fig. 9). Furthermore, we observed that treatment of TC-1S cells with either CNTF or IL-6 increased the number of NF$^+$ cells by 2.5–3 fold, respectively (Fig. 10 *A*). Both IL-6 and CNTF were also able to increase NF mRNA levels (by 6-fold and 10-fold, respectively) (Fig. 10 *B*).

Discussion

The coexistence of migrating neural crest-derived elements or cells committed to the neuronal phenotype together with distinct cell lineages appears to be widespread throughout several tissues (e.g., gut, thyroid, pancreas), including the thymus (2, 33, 34, 44). Interestingly, in this organ a tight link between epithelial and neural cell lineages has been suggested by the observation that neuronal cell cytoskeletal expression is confined to cytokeratin-positive epithelial cells in cortical-type epithelial thymomas (37). The coexpression of features characteristic of both epithelial and neuronal differentiation which has also been described in some neuroendocrine or neuroectoderm-derived tumors (6) suggests the existence of processes underlying epithelial-neural transition. The mechanisms involved in the regulation of these processes are likely to require the local production of factors with multifunctional properties with regard to neural and other cell lineage trophisms. The choice between the different cell fates may thus be defined by the balance of distinct growth factors. This has been, for instance, described for NGF and glucocorticoids which have been previously reported to be the major regulators of the choice of the neural crest-derived cells towards the endocrine or sympathetic neuronal fate (reviewed in reference 2).

The involvement of EGF-like–related signals in determining the choices between neuronal and epithelial cell

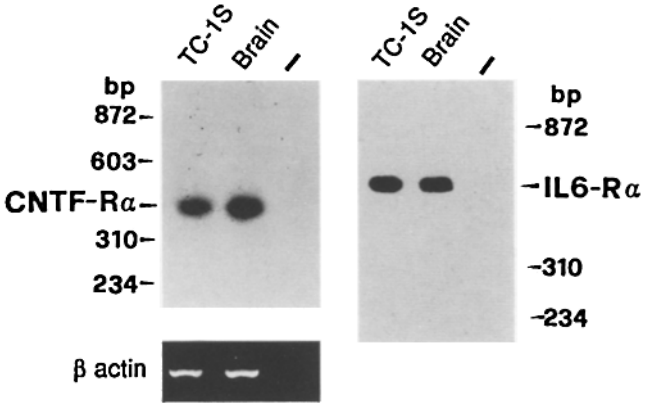

Figure 9. Expression of CNTF and IL-6 receptors α subunits in TC-1S cells. RT-PCR of CNTFRα and IL-6Rα transcripts in RNA from TC-1S cells and mouse brain. Negative control with no RNA added to the RT-PCR reaction is also shown. Southern hybridization of amplified products using the described 30-mer internal probes is shown. Ethidium bromide staining of β-actin amplification, as an internal control, is also shown.

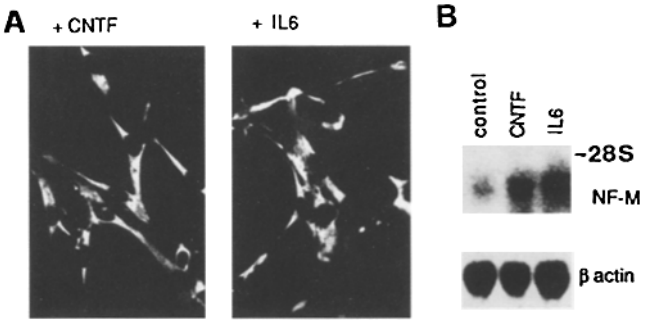

Figure 10. CNTF and IL-6 enhance neuronal phenotype in TC-1S cells. (*A*) Fluorescence microphotograph of NF-immunoreactive TC-1S cells 48 h after either CNTF or IL-6 treatment. (*B*) Northern blot analysis of NF-M mRNA levels in untreated TC-1S cells (*control*) and 48 h after either CNTF or IL-6 treatment.

fate is suggested by the ability of the Drosophila *Notch* gene product, which harbors sequence elements homologous to a region of EGF, to segregate epidermal and neuronal lineages from the ventral neuroectoderm and to further regulate the coexistence of both cell types (reviewed in 3).

In this paper we report that EGF itself is able to control epithelial–neural cell relationships, as it behaves as a major autocrine and/or paracrine enhancer of neural cell type distribution in several thymic epithelial cell cultures. We also show that EGF was able to promote a multivalent neurotypic response characterized by (*a*) increased proliferation and differentiation of cells expressing neuronal phenotype; (*b*) enhancement of cells with specific neuronal function (e.g., expression of substance P neurotransmitter); and (*c*) enhancement of specific neurotrophic factors. Thus we confirm the recently reported neuropoietic effect of EGF on neuronal precursors of the CNS (45, 59) and extend these observations to include an effect on distinct cell lineages. The ability of EGF to promote both the proliferation and the neuronal differentiation we have observed in thymic epithelial cells, is similar to the effects on cells of the sympathoadrenal lineage: both effects have been, in fact, described in rat pheochromocytoma PC12 cells (26, 40). The neuropoietic potential of EGF has been also observed on cells at a less differentiated stage, as neuronal differentiation of undifferentiated P19 embryonal carcinoma cells, in response to this growth factor, has been reported (13).

We have further characterized the EGF response with regard to neuropeptide and neurotrophic factor regulation. One of the most striking aspects of our study concerns the EGF-mediated modulation of CNTF and IL-6 expression in thymic epithelial cells and their potential role in contributing, together with EGF, to neuropoiesis within this cell population. The contribution of IL-6 and CNTF to the neuropoietic activity of EGF was suggested by the early induction of these cytokines by EGF and the subsequent enhancement of the neural phenotype. Indeed, the addition of exogenous IL-6 and CNTF promoted the neural phenotype in thymic epithelial cell cultures.

To our knowledge, CNTF regulation by EGF has not previously been reported. Both EGF and CNTF and other gp130-transducing signals have been reported to enhance the same *cis*-regulatory elements of responsive genes through the activation of transacting nuclear factors belonging to the STAT family (17, 27, 48, 49, 53, 60). This, together with the observation that EGF regulates CNTF expression, suggests that both factors extensively interact at multiple levels.

The signals that modulate CNTF expression remain largely unidentified. EGF, which enhances wound healing and tissue regeneration (10), may link such processes to CNTF expression. Indeed, CNTF prevents retrograde neuronal death after axotomy and alteration of the levels of this cytokine have been reported during nerve degeneration and regeneration (11, 22, 23, 28, 52). The presence of a functional CNTF/CNTF-receptor pathway and its possible participation in the neuropoietic response induced by EGF, extends the panel of CNTF target cells and is in keeping with a more general role for this cytokine in the growth control and maturation of neuronal precursors. This is further supported by the expression of CNTF and CNTF-Rα in embryonic tissues previously reported (29).

The discovery of a functionally active neurotrophic microenvironment within the thymus and the link between epithelial and neural cells are intriguing considering that relationships between the nervous and immune systems and their deregulation have been implicated in the pathogenesis of several autoimmune diseases (e.g., myasthenia gravis associated with thymic epithelial cell tumors and autoimmunity against axons and other neural structures, insulin-dependent diabetes mellitus) (5, 30, 31, 37, 58). The neuromodulatory role of EGF in the thymus reveals a novel characteristic of this growth factor in the relationship between neural networks and the immune system. We suggest that EGF may play a critical role in directing thymic epithelial cells towards a neural-oriented cell fate, a phenomenon characteristic of certain myasthenia gravis-associated thymomas (37). The growth factor might thus be involved in the pathogenesis of thymic epithelial tu-

mors and the subsequent development of autoimmunity. Moreover, the EGF-regulated bipotential neuro- and lymphopoietic cytokines (CNTF and IL-6) or the growth factor itself might either directly influence thymic lymphoid cells or sustain an appropriate intrathymic neuronal cell population and neurotransmitter production, which in turn may affect thymocyte development. In this respect, several neurotransmitters and neuropeptides (including norepinephrine, acetylcholine and substance P) have been shown to regulate the function of mature and developing lymphocytes (9, 39, 46, 54). Interestingly, we have observed that EGF-treated thymic epithelial cells reduce the ability of $CD4^-8^-$precursor thymocytes to generate more mature $CD4^+8^+$ T cells in lympho-epithelial cell cocultures in vitro (our unpublished data). Thus, the composite spectrum of EGF activity upon the thymus suggests that this organ may be the site at which a novel regulatory activity of the growth factor upon neural-immune interactions could occur.

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References

1. Anchan, R. M., T. A. Reh, J. Angello, A. Balliet, and M. Walker. 1991. EGF and TGF-α stimulate retinal neuroepithelial cell proliferation in vitro. *Neuron.* 6:923–936.
2. Anderson, D. J. 1993. Molecular control of cell fate in the neural crest: the sympathoadrenal lineage. *Annu. Rev. Neurosci.* 16:129–158.
3. Artavanis-Tsakonas, S., and P. Simpson. 1991. Choosing a cell fate: a view from the Notch locus. *Trends Genet.* 11/12:403–406.
4. Auerbach, R. 1960. Morphogenetic interactions in the development of the mouse thymus gland. *Dev. Biol.* 2:271–279.
5. Baekkeskov, S., H.-J. Aanstoot, S. Christgau, A. Reetz, M. Solimena, M. Cascalho, F. Folli, H. Richter-Olesen, and P.-D. Camilli. 1990. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature (Lond.).* 347: 151–156.
6. Baudoin, C., G. Meneguzzi, M. M. Portier, M. Demarchez, F. Bernerd, A. Pisani, and J. P. Ortonne. 1993. Peripherin, a neuronal intermediate protein, is stably expressed by neuroendocrine carcinomas of the skin, their xenograft on nude mice, and the corresponding primary cultures. *Cancer Res.* 53:1175–1184.
7. Bockman, D. E., and M. L. Kirby. 1984. Dependence of thymus development on derivatives of the neural crest. *Science (Wash. DC).* 223:498–500.
8. Boyd, R. L., C. L. Tucek, D. I. Godfrey, D. J. Izon, T. J. Wilson, N. J. Davidson, A. G. D. Bean, H. M. Ladyman, M. A. Ritter, and P. Hugo. 1993. The thymic microenvironment. *Immunol. Today.* 14:445–448.
9. Calvo, C. F., G. Chavanel, and A. Senik. 1992. Substance P enhances IL-2 expression in activated human T cells. *J. Immunol.* 148:3498–3504.
10. Carpenter, G., and M. I. Wahl. 1990. Peptide Growth Factors and their Receptors. M. B. Sporn and A. B. Roberts, editors. Springer-Verlag, Berlin. 69–171.
11. Clatterbuck, R. E., D. L. Price, and V. E. Koliatsos. 1993. Ciliary neurotrophic factor prevents retrograde neuronal death in the adult central nervous system. *Proc. Natl. Acad. Sci. USA.* 90:2222–2226.
12. Davis, S., T. H. Aldrich, N. Stahl, L. Pan, T. Taga, T. Kishimoto, N. Y. Ip, and G.D. Yancopoulos. 1993. LIFRβ and gp 130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science (Wash. DC).* 260: 1805–1808.
13. den Hertog, J., S. W. de Laat, J. Schlessinger, and W. Kruijer. 1991. Neuronal differentiation in response to epidermal growth factor of transformed murine P19 embryonal carcinoma cells expressing human epidermal growth factor receptors. *Cell Growth & Differ.* 2:155–166.
14. Ericsson, A., V. Geenen, F. Robert, J.-J. Legros, Y. Vrindts-Gevaert, P. Franchimont, S. Brene, and H. Persson. 1990. Expression of Preprotachykinin-A and Neuropeptide-Y messenger RNA in the thymus. *Mol. Endocrinol.* 4:1211–1218.
15. Fallon, J. H., K. B. Seroogy, S. E. Loughlin, R. S. Morrison, R. A. Bradshaw, D. J. Knauer, and D. D. Cunningham. 1984. Epidermal growth factor immunoreactive material in the central nervous system: location and development. *Science (Wash. DC).* 224:1107–1110.
16. Friedman, B., S. S. Scherer, J. S. Rudge, M. Helgren, D. Morrisey, J. McClain, D.-Y. Wang, S. J. Wiegand, M. E. Furth, R. M. Lindsay, and N. Y. Ip. 1992. Regulation of ciliary neurotrophic factor expression in myelin-related Schwann cells in vivo. *Neuron.* 9:295–305.
17. Fu, X.-Y., and J.-J. Zhang. 1993. Transcription factor p91 interacts with the epidermal growth factor receptor and mediates activation of the c-fos gene promoter. *Cell.* 74:1135–1144.
18. Fuller, P. J., and K. Verity. 1989. Somatostatin gene expression in the thymus gland. *J. Immunol.* 143:1015–1017.
19. Geenen, V., J.-J. Legros, P. Franchimont, M. Baudrihaye, M.-P. Defresne, and J. Boniver. 1986. The neuroendocrine thymus: coexistence of oxytocin and neurophysin in the human thymus. *Science (Wash. DC).* 232:508–511.
20. Geenen, V., F. Robert, M. Fatemi, H. Martens, M.-P. Defresne, J. Boniver, J.J. Legros, and P. Franchimont. 1989. Neuroendocrine-immune interactions in T cell ontogeny. *Thymus.* 13:131–140.
21. Gomez-Pinilla, F., D. J. Knauer, and M. Nieto-Sampedro. 1988. Epidermal growth factor receptor immunoreactivity in rat brain. Development and cellular localization. *Brain Res.* 438:385–392.
22. Hagg, T., and S. Varon. 1993. Ciliary neurotrophic factor prevents degeneration of adult rat substantia nigra dopaminergic neurons in vivo. *Proc. Natl. Acad. Sci. USA.* 90:6315–6318.
23. Hagg, T., D. Quon, J. Higaki, and S. Varon. 1992. Ciliary neurotrophic factor prevents neuronal degeneration and promotes low affinity NGF receptor expression in the adult rat CNS. *Neuron.* 8:145–156.
24. Hall, A. K., and M. S. Rao. 1992. Cytokines and neurokines: related ligand and related receptors. *Trends Neurosci.* 15:35–37.
25. Huff, K., D. End, and G. Guroff. 1981. Nerve growth factor-induced alteration in response of PC12 pheochromocytoma cell to epidermal growth factor. *J. Cell Biol.* 88:189–198.
26. Hirokawa, K., M. Utsuyama, M. Kasai, A. Konno, C. Kurashima, and E. Moriizumi. 1990. Age-related hyperplasia of the thymus and T-cell system in the Buffalo rat. *Virchows Archiv. B Cell Pathol.* 59:38.
27. Ihile J. N., A. B. Witthuhn, F. W. Qelle, K. Yamamoto, W. E. Thierfelder, B. Kreider, and O. Silvennoinen. 1994. Signaling by the cytokine receptor superfamily: JAKs and STATs. *Trends Biochem. Sci.* 19:222–225.
28. Ip, N. Y., S. J. Wiegand, J. Morse, and J. S. Rudge. 1993. Injury-induced regulation of ciliary neurotrophic factor mRNA in the adult rat brain. *Eur. J. Neurosci.* 5:25–33.
29. Ip, N. Y., J. McClain, N. X. Barrezueta, T. H. Aldrich, L. Pan, Y. Li, S. J. Wiegand, B. Friedman, S. Davis, and G. D. Yancopoulos. 1993. The α component of the CNTF receptor is required for signaling and defines potential CNTF targets in the adult and during development. *Neuron.* 10: 89–102.
30. Kao, I., and D. B. Drachman. 1977. Thymic muscle cells bear acetylcholine receptors: possible relation to myasthenia gravis. *Science (Wash. DC).* 195:74–77.
31. Kaufman, D.L., M. Clear-Salzler, J. Tian, T. Fursthuber, G. S. P. Ting, P. Robinson, M. Atkinson, E. E. Sercarz, A. J. Tobin, and P. V. Lehmann. 1993. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature (Lond.).* 366: 69–72.
32. Le, P. T., S. Lazorich, L. P. Whichard, B. F. Haynes, and K. H. Singer. 1991. Regulation of cytokine production in the human thymus: epidermal growth factor and transforming growth factor a regulate mRNA levels of interleukin 1α (IL-1α), IL-1β, and IL-6 in human thymic epithelial cells at a post-transcriptional level. *J. Exp. Med.* 174:1147–1157.
33. Le Douarin, N. M. 1982. The Neural Crest. Cambridge University Press.
34. Le Douarin, N., C. Dulac, E. Dupin, and P. Cameron-Curry. 1991. Glial cell lineages in the neural crest. *Glia.* 4:175–184.
35. Le Lièvre, C., and N. Le Douarin. 1974. Rôle des cellules mèsectodermiques issues des crêtes neurales cèphaliques dans la formation des arcs branchiaux et du squelette viscèral. *J. Embryol. Exp. Morphol.* 31:453–477.
36. Lobach, D. F., and B. F. Haynes. 1987. Ontogeny of the human thymus during fetal development. *J. Clin. Immunol.* 7:81–97.
37. Marx, A., T. Kirchner, A. Greiner, H. K. Muller-Hemerlink, B. Schalke, and M. Osborn. 1992. Neurofilament epitopes in thymoma and antiaxonal autoantibodies in myasthenia gravis. *Lancet.* 339:707–708.
38. Meco, D., S. Scarpa, M. Napolitano, D. Bellavia, M. Ragano-Caracciolo, A. Modesti, L. Frati, A. Gulino, and I. Screpanti. 1994. Modulation of fibronectin and thymic stromal cell-dependent thymocyte maturation by retinoic acid. *J. Immunol.* 153:73–83.
39. Mihovilovic, M., and A. D. Roses. 1993. Expression of α-3, α-5, and β-4 neuronal acetylcholine receptor subunit transcripts in normal and myasthenia gravis thymus. *J. Immunol.* 151:6517–6524.
40. Morrison, R. S., H. I. Kornblum, F. M. Leslie, and R. A. Bradshaw. 1987. Trophic stimulation of cultured neurons from neonatal rat brain by epidermal growth factor. *Science (Wash. DC).* 238:72–75.
41. Nakafuku, M., and Y. Kaziro. 1993. Epidermal growth factor and trans-

forming growth factor-α can induce neuronal differentiation of rat pheochromocytoma PC12 cells under particular culture conditions. *FEBS (Fed. Eur. Biochem Soc.) Lett.* 315:227–232.
42. Nakamura, H., and C. Ayer-Le Lièvre. 1986. Neural crest and thymic myoid cells. *Curr. Top. Dev. Biol.* 20:111–115.
43. Patterson, P. H., and H. Nawa. 1993. Neuronal differentiation factors/cytokines and synaptic plasticity. *Cell/Neuron.* 72/10:123–137.
44. Polak, M., R. Scharfmann, B. Seilheimer, G. Eisenbarth, D. Dressler, I. M. Verma, and H. Potter. 1993. Nerve growth factor induces neuron-like differentiation of an insulin-secreting pancreatic beta cell line. *Proc. Natl. Acad. Sci. USA.* 90:5781–5785.
45. Reynolds, B. A., and S. Weiss. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science (Wash. DC).*255:1707–1710.
46. Richman, D. P., and B. G. W. Arnason. 1979. Nicotinic acetylcholine receptor: evidence for a functionally distinct receptor on human lymphocytes. *Proc. Natl. Acad. Sci. USA.* 76:4632–4635.
47. Ritter, M. A., and R. L. Boyd. 1993. Development in the thymus: it takes two to tango. *Immunol. Today.* 14:462–465.
48. Ruff-Jamison, S., K. Chen, and S. Cohen. 1993. Induction by EGF and interferon-γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. *Science (Wash. DC).* 261:1733–1736.
49. Sadowski, H. B., K. Shuai, J. E. Darnell, Jr., and M. Z. Gilman. 1993. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science (Wash. DC).* 261:1739–1741.
50. Screpanti, I., D. Meco, S. Scarpa, S. Morrone, L. Frati, A. Gulino, and A. Modesti. 1992. Neuromodulatory loop mediated by nerve growth factor and interleukin 6 in the thymic stromal cell cultures. *Proc. Natl. Acad. Sci. USA.* 89:3209–3212.
51. Screpanti, I., A. Modesti, and A. Gulino. 1993. Heterogeneity of thymic stromal cells and thymocyte differentiation: a cell culture approach. *J. Cell Sci.* 105:601–606.
52. Sendtner, M., G. W. Kreutzberg, and H. Thoenen. 1990. Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature (Lond.).*345:440–442.
53. Silvennoinen, O., C. Schindler, J. Schlessinger, and D. E. Levy. 1993. Ras-independent growth factor signaling by transcription factor tyrosine phosphorylation. *Science (Wash. DC).* 261:1736–1739.
54. Singh, U., D. S. Millson, P. A. Smith, and J. J. T. Owen. 1979. Identification of β adrenoreceptors during thymocyte ontogeny in mice. *Eur. J. Immunol.* 9:31–36.
55. Stemple, D. L., and D. J. Anderson. 1992. Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell.* 71:973–982.
56. Stockli, K. A., L. E. Lillien, M. Naher-Noè, G. Breitfeld, R. A. Hughes, M. C. Raff, H. Thoenen, and M. Sendtner. 1991. Regional distribution developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. *J. Cell Biol.* 115:447–459.
57. Tanabe, O., S. Akira, T. Kamiya, G. G. Wong, T. Hirano, and T. Kishimoto. 1988. Genomic structure of the murine IL-6. *J. Immunol.* 141. 3875–3883.
58. Tisch, R., X. D. Yang, S. M. Singer, R. S. Liblau, L. Fuger, and H. O. McDevitt. 1993. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature (Lond.).* 366:72–75.
59. Vescovi, A. L., B. A. Reynolds, D. D. Fraser, and S. Weiss. 1993. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron.* 11· 951–966.
60. Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1994. Stat 3: a stat family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science (Wash. DC).* 264:95–98.