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.

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The Journal of Cell Biology, Volume 130, Number 1, July 1995 183-192

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 10⁶ 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 Neu200; from Boehringer Mannheim GmbH, Mannheim, Germany), anti-NF 160 kD (Amersham Corp., Buckinghamshire, UK), pan-cytokeratin monoclonal antibody (clone L5, 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) fluorences 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 [125I]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 Amer sham 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, dehydrated 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 hematoxalin. 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 MgCl₂, 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. 5'-actin internal control for both reverse transcription and the PCR. The 5' and 3' CNTF primers were 5'-CCAGTGGCAAGCACTGATC-3' and 5'-CCCATAATGCTCTTCACAGTC-3', respectively. The 5' and 3' CNTF-Ra primers were 5'-GCTGTCACATTCGGTACATGC-3' and 5'-AGCCA- CATCCGCAGTCACCTCA-3', respectively. The 5' and 3' IL-6-Ra primers were 5'-TCTATGATGTCACGCACGAT-3' and 5'-TATI'TGATGTCACGCACGATI'TC-3' as 5' and 3' primers, served as an internal control for both reverse transcription and the PCR.

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 125I-EGF with a Kᵦ 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-M (NF-M)-specific 32P-labeled probe (pNF36; American Type Culture Collection) (top) and, after stripping, a β-actin probe (bottom). 28S RNA 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 cell population in the absence of EGF (-EGF) or 48 h after the addition of the growth factor (+EGF) in several murine TC cultures.
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>
<thead>
<tr>
<th>Human cell line</th>
<th>Control</th>
<th>EGF</th>
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<tr>
<td></td>
<td>NF</td>
<td>TH</td>
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<tr>
<td>HTC-3</td>
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<td>8 ± 1.5</td>
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<tr>
<td>HTC-19</td>
<td>16 ± 1.5</td>
<td>5 ± 2</td>
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Table I. Percent (Mean ± SD) of NF or TH-positive or SYN-positive Cells in Human TC Cultures before (Control) and after EGF Treatment.
EGF also enhanced the expression of other markers specific for neuronal and/or neuroendocrine cells including synapsin I and tyrosine hydroxylase (TH) (Fig. 3, e, f, h, and i). The percentage of TH$^+$ cells increased from $9 \pm 2\%$ to $35 \pm 4\%$ and that of synapsin$^+$ (Syn) cells from $7 \pm 2\%$ up to $30 \pm 6\%$, 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...
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 expression of IL-6 and CNTF 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).
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'-TAATCCAAAGAACTGCTGAG-GCTYGGGTCT-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).

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
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 neurotrophic 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 potential of EGF 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 autoimmune 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 noradrenaline, 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 interaction could occur.

We thank Drs. F. Eusebi, M. Israel and A.R. Mackay for critical reading of the manuscript. We are grateful to Dr. R. De Maria for help in the cell-cycle study and Dr. A. Stoppacciaro for immunoperoxidase staining of cryo-sections.

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