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Expression of Growth Factors and Growth Factor Receptors in Normal and Tumorous Human Thyroid Tissues

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ABSTRACT

A number of growth factors have been implicated as stimuli of thyroid cell proliferation; overexpression of these growth factors and/or their receptors may play a role in the growth of thyroid tumors. To determine if immunohistochemical detection of growth factors and/or their receptors correlates with morphological alterations in proliferative lesions of thyroid, we examined the localization of epidermal growth factor (EGF), transforming growth factor- α (TGF- α) and their common receptor, EGF-receptor (EGF-R), insulin-like growth factor-1 (IGF-1), IGF-1-receptor (IGF-R) and IGF binding proteins (IGFBP)-1, -2, -3, and -4, nerve growth factor (NGF), and its receptor NGF-receptor (NGF-R), transforming growth factor- β (TGF- β), and basic fibroblast growth factor (bFGF), in normal thyroid tissue and various thyroid tumors. We applied the streptavidin-biotin technique to formalin-fixed, paraffin-embedded tissues. We studied 8–16 different cases of each of the following: normal human thyroid, multinodular hyperplasia, follicular adenoma, papillary carcinoma, follicular carcinoma, medullary carcinoma, and anaplastic carcinoma. EGF, TGF- α , and their receptor EGF-R were widely expressed in normal thyroid and in all the thyroid lesions examined. IGF-1 and IGFBP-1 were diffusely present in all different thyroid tissues as well. There was no difference in staining intensity or distribution that correlated with the pathological process. IGFBP-4 seemed to have a variable expression. IGFBP-2 and -3 were detected only in medullary carcinomas. NGF immunoreactivity was found in thyroid tissues and tumors of all types; interestingly, NGF-R staining was found in the vascular stroma and immunoreactivity correlated with the degree of vascularization, but no staining was seen in normal or lesional thyroid cells. TGF- β expression was highly variable, whereas bFGF was not detected by this method in thyroid tissues. This study indicates that growth factors and growth factor receptors are expressed by thyroid follicular cells and C-cells. While they most likely play a role in cell regulation and proliferation in these tissues, their immunohistochemical profile does not correlate with the pathological condition.

INTRODUCTION

THE GROWTH OF NORMAL CELLS is controlled by stimulating and inhibiting factors. Growth factors are polypeptides that regulate the replication and differentiated function of cells. They exert their effect by interaction with specific surface receptors on responsive cells. Some are mitogenic, such as epidermal growth factor (EGF) and transforming growth factor- α (TGF- α); others, such as transforming growth factor- β (TGF- β), specifically inhibit cell growth and may play a role in regulating differentiation (1,2).

Thyrocytes, like other cells, respond *in vitro* to paracrine or autocrine secretion of growth factors (3). A number of growth factors have been shown to stimulate function and growth of

thyroid tissue and thus play a role in thyroid cell growth in non-neoplastic conditions, such as hyperplastic goiter, and in benign and malignant neoplasms. It has been suggested that excessive systemic or autocrine production of a growth factor can cause uncontrolled cell replication. The presence of an oncogene encoding an abnormal growth factor receptor on the cell surface can also cause excessive cell replication, even in the absence of the corresponding growth factor ligand. All these mechanisms can lead to cell transformation and the development of neoplasia.

The thyroid provides a convenient model for studying the expression of growth factors in human epithelial tumors. It provides a well-defined spectrum of hyperplasias, benign and malignant neoplasms. Previous studies have suggested that certain

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growth factors may be important mediators of cell proliferation in the thyroid (3,4). To define the localization of various growth factors and their putative receptors, and to determine if their detection correlates with the pathophysiological process, we studied their immunohistochemical localization in normal thyroid tissue as well as in a variety of benign and malignant thyroid lesions.

MATERIALS AND METHODS

Case material

Material was retrieved from the surgical pathology and autopsy files of a consultation practice in thyroid pathology (S.L.A.). All tissues had been fixed in formalin and embedded in paraffin. For tissue and tumor classification, sections were stained with hematoxylin–eosin and a variety of immunohistochemical stains; all lesions were classified using the criteria provided by Murray (5) and LiVolsi (6); only classical examples of each pathological process were used for this study and any controversial cases were excluded.

Selection of material for this study was based primarily on diagnosis and included tumors and nontumorous tissues from patients of various ages and both sexes to obtain appropriate numbers of each tissue and tumor type.

Immunohistochemistry

Immunostaining was performed on 4- to 6- μ m-thick sections using the avidin–biotin–peroxidase complex technique. All immunostains were performed in batches using the same reagents to allow semiquantitative analysis of the results. Primary antibodies and antisera were used to detect the following antigens at the specified dilutions: polyclonal antibody to EGF (Collaborative Biomedical Products) 1:200; monoclonal antibody to TGF- α (Oncogene Science) 1:100; polyclonal antiserum to IGF-1 (NIDDK) 1:2000; polyclonal antiserum to NGF (Serotec) 1:50; polyclonal antiserum to TGF- β (Collaborative Biomedical Products) 1:100; polyclonal antiserum to bFGF (R & D System) 1:50 and monoclonal antibody to bFGF (Upstate Biotechnology Inc.) 1:500; monoclonal antibody to EGF-R (Biogenex) 1:50; monoclonal antibody to NGF-R (Amersham Life Science) 1:30; polyclonal antiserum to IGF-R (Upstate Biotechnology Inc.) 1:1000 and polyclonal antisera to IGF-BPs-1, -2, -3, and -4 (Upstate Biotechnology Inc.) 1:1500, 1:1500, 1:500, and 1:200, respectively.

Positive controls for each procedure were obtained using the following tissues: for EGF, human placenta; for TGF- α and bFGF, bovine pituitary gland; for IGF-1, human liver and human pituitary gland; for NGF, submandibular gland; for TGF- β , human small bowel; for EGF-R, human skin; for IGF-R, human pituitary; for IGF-BP-1, human endometrium; for IGF-BP-2, human pituitary; for IGF-BP-3, human liver; and for IGF-BP-4, human kidney. For all immunostaining, pretreatment of tissue sections was performed with pronase, pepsin, protease type XXIV, saponin, or microwave antigen retrieval to enhance positivity. Pronase was applied to sections stained for bFGF, pepsin predigestion was used prior to staining for EGF-R, NGF-R, IGF-R, and all IGF-BPs, and saponin was applied to all sections stained for TGF- α .

The specificity of immunostaining was verified by absorption of primary antibody with excess homologous and heterologous antigens. In all cases except one, homologous antigen absorption totally abolished staining. In the case of NGF, partial but not total negativity was obtained with up to 100 μ g purified 2.5S NGF/mL antiserum. Heterologous antigen had no effect on staining.

Quantitation

The degree of positivity for each stain on each tissue was graded as follows: 0, no positivity; 1+, <1/3 of cells positive; 2+, 1/3–2/3 of cells positive; and 3+, >2/3 of cells positive. The intensity of positive staining was also graded on a scale of 0–3+ where 0 was negative and 3+ was very intense staining.

RESULTS

The tissues examined and the results of immunohistochemical staining are summarized in Tables 1 and 2. The table indicates only those tissues that were positive or negative, but because of the variability of degree and intensity of staining, it does not include the results of semiquantitative analysis; this information is detailed in the following paragraphs.

EGF was localized in 32 of 44 normal thyroids, 11/11 nodular hyperplasias, 7/10 follicular adenomas (Fig. 1), 12/13 papillary carcinomas, 6/7 follicular carcinomas, 9/9 medullary carcinomas, and in both anaplastic carcinomas examined. The staining was diffuse in the cell cytoplasm and generally strong in intensity, ranging from 2+ to 3+; in most cases the distribution of positivity was widespread throughout normal and le-

TABLE 1. IMMUNOHISTOCHEMICAL PROFILE OF GROWTH FACTORS IN THYROID TISSUE AND TUMORS^a

	EGF	TGF- α	IGF-1	NGF	TGF- β	bFGF
Normal	+	+	+	+/-	+/-	-
Hyperplasia	+	+	+	+	+/-	-
Adenoma	+	+	+	+	+/-	-
Papillary ca	+	+	+	+	+/-	-
Follicular ca	+	+	+	+/-	+/-	-
Medullary ca	+	+	+	+	+/-	-
Anaplastic ca	+	+	+	+	+	-

^a+, positive staining; -, no staining; +/-, variable staining.

TABLE 2. IMMUNOHISTOCHEMICAL PROFILE OF GROWTH FACTOR RECEPTORS AND BINDING PROTEINS IN THYROID TISSUE AND TUMORS^a

	<i>EGF-R</i>	<i>NGF-R^b</i>	<i>IGF-R</i>	<i>IGFBP-1</i>	<i>IGFBP-2</i>	<i>IGFBP-3</i>	<i>IGFBP-4</i>
Normal	+	—	—	+/-	—	—	+/-
Hyperplasia	+	—	—	+/-	—	—	+/-
Adenoma	+	—	—	+/-	—	—	+/-
Papillary ca	+	—	—	+/-	—	—	—
Follicular ca	+	—	—	+/-	—	—	—
Medullary ca	+	—	—	+	+	+	+
Anaplastic ca	+	—	—	+/-	—	—	+

^a+, positive staining; —, no staining; +/-, variable staining.
^bLocalized in vessel walls but not in thyroid cells.

sional tissue with more than 50% of cells positively stained. There was no difference in the number of positive cells or the staining intensity in lesional tissue compared with the surrounding nontumorous tissue in each given specimen nor was there any statistically significant difference in the distribution or intensity of staining between tumor groups.

TGF- α was detected in almost all normal tissues (50/54), in all 15 nodular hyperplasias, in 10/11 follicular adenomas, 15/16 papillary carcinomas (Fig. 2), 8/9 follicular carcinomas, 8/8 medullary carcinomas, and both anaplastic tumors. The staining was diffuse in the cell cytoplasm; again, the distribution was widespread throughout the majority of cells in each lesion and the intensity was strong, graded as 3+ in most cases. There was no difference in the number of positive cells or the staining intensity in lesional tissue compared with the surrounding nontumorous tissue in each given specimen nor was there any statistically significant difference in the distribution or intensity of staining between tumor groups.

The putative receptor for EGF and TGF- α (EGF-R) was found in a membrane distribution in the normal tissue of 24/29 patients. All 15 nodular hyperplasias, 8/10 follicular adenomas, 11/12 papillary carcinomas (Fig. 3), all 6 follicular carcinomas, 4/9 medullary carcinomas, and both anaplastic carcinomas ex-

hibited EGF-R. The number of positive cells was highly variable, ranging from 1+ to 3+ in normal and lesional tissues. The staining intensity was generally 1+ to 2+. In one follicular adenoma, one follicular variant papillary carcinoma (Fig. 3), and both anaplastic carcinomas the positivity was very intense, graded as 3+, and was present in virtually all the cells included in the sections examined; this pattern of staining was distinctly stronger and more diffuse than in the surrounding nonlesional tissue in these cases. Nevertheless, this pattern was not seen consistently in other neoplasms.

Immunoreactive IGF-1 was found in the normal thyroid tissue of 32 patients; another 9 cases were entirely negative. All 13 nodular hyperplasias, all 9 follicular adenomas (Fig. 4), all 13 papillary carcinomas, 7/8 follicular carcinomas, all 8 medullary carcinomas, and both anaplastic cancers had diffuse strong cytoplasmic staining that was graded as 2+ to 3+ intensity in most cases; the intensity was similar in lesional and nonlesional tissue of individual specimens and did not correlate with the diagnosis or behavior of the various lesions. The number of positive cells, however, did differ in that lesions were usually composed almost exclusively of positive cells (3+ staining) whereas staining in the normal tissue was focal (1+ to 2+) and many cells did not contain immunoreactivity for IGF-1.

FIG. 1. A follicular adenoma contains diffuse strong cytoplasmic positivity for epidermal growth factor (top left); a similar staining pattern is seen in the cells of the surrounding nontumorous thyroid (right) (magnification $\times 25$).

FIG. 2. Immunoreactivity for TGF- α is strong in the cytoplasm of tumor cells in a papillary thyroid carcinoma; the fibrous stroma is negative (magnification $\times 25$).

FIG. 3. The staining intensity for EGF-R in this follicular variant papillary carcinoma is very intense, graded as 3+, and present in virtually all the cells (magnification $\times 50$).

FIG. 5. Strong staining for IGFBP-2 is localized in scattered cells of a medullary carcinoma (magnification $\times 50$).

IGF-R could not be localized in any thyroid tissue examined, despite strong and reproducible positivity in human pituitary glands. IGFBP-1 was detected in a few glands in normal tissue, in some nodular hyperplasias, and in some examples of each of the tumor types; while some specimens of each tumor type stained, others were entirely negative and the only consistent result was the positivity of all 10 medullary carcinomas that exhibited 2+ to 3+ intensity of staining in the majority of cells. IGFBP-2 and -3 were only localized in 10/10 and 9/10 medullary carcinomas, respectively, also with strong staining in a significant number of scattered cells (Fig. 5); the other thyroid tumors and normal thyroid tissues were entirely negative. IGFBP-4 was detected in some normal tissues and in a few examples of nodular hyperplasia and follicular adenoma; well-differentiated thyroid carcinomas of follicular cell differentiation were all negative. Medullary carcinomas consistently exhibited weak staining (1+ to 2+) for this binding protein in variable numbers of cells. The two anaplastic carcinomas were

strongly positive in the majority of cells (3+ distribution, 3+ intensity).

Stains to localize NGF were positive in 19/34 normal glands, 8/9 nodular hyperplasias, 8/9 follicular adenomas, 8/10 papillary carcinomas, 6/8 follicular carcinomas, 8/9 medullary carcinomas, and both anaplastic tumors. The staining was variably in intensity, mainly 2–3+ but in some cases weak (1+), and was diffuse throughout the cytoplasm of the majority of tumor and nontumorous cells. In contrast, the putative receptor for NGF (NGF-R) was not found in thyroid follicular epithelium, in C-cells, or in their proliferative lesions. NGF-R positivity was very intense around blood vessels in normal thyroid, however, no staining was seen in the stroma of thyroid lesions except around large vessels.

TGF- β staining was positive in 10/43 normal glands, 8/13 nodular hyperplasias, 5/11 follicular adenomas, 4/11 papillary carcinomas, 4/7 follicular carcinomas, and 6/8 medullary carcinomas. The staining was rather weak in intensity, graded as 1+ in most cases and 2+ at maximum. Both anaplastic carcinomas had faint immunoreactivity. The number of positive cells in lesions and nontumorous thyroid was highly variable and there was no correlation between staining and the tissue morphology.

bFGF could not be localized in any normal thyroid tissue; staining was found in a single case of nodular hyperplasia and in one papillary carcinoma. The positivity was very focal, suggesting clonal expression of this factor. Careful absorption studies confirmed the specificity of this finding.

DISCUSSION

Although the size and function of the thyroid gland are mainly controlled by thyroid stimulating hormone (TSH), iodide and growth hormone (GH) (3), extrapituitary controls also exist, as proven by compensatory hyperplasia of the gland after hemithyroidectomy in hypopituitary or hypophysectomized animals (7). Moreover, patients with autoimmune thyroid disease exhibit thyroid proliferation that is clearly TSH-independent. The main factors implicated in the extrapituitary control

FIG. 4. Immunoreactive IGF-I is found in a follicular adenoma and the surrounding normal thyroid tissue; the staining is diffuse and strong in the cell cytoplasm, and similar in lesional and non-lesional tissue (magnification $\times 25$).

of thyroid cell proliferation are thyroid stimulating immunoglobulins (TSIs), cytokines, and growth factors (3,4).

Although controversial, EGF immunoreactivity and mRNA have been detected in thyroid tissue (8–11). No significant difference in the pattern of immunoreactivity was detected between benign and malignant lesions, but the frequency of recurrence of papillary cancer was reported to correlate with intensity of EGF staining (10). In contrast, no EGF was detected in conditioned media from a human anaplastic thyroid carcinoma cell line (12). We found EGF in all thyroid tissues examined, and we were unable to correlate the intensity of staining with biological behavior, suggesting that this technique is not a useful predictive marker in tumor diagnosis. Nevertheless, EGF stimulates follicular cell proliferation and reduces function and/or differentiation (13–21) and may therefore act as a promoter in thyroid tumorigenesis.

Transforming growth factor- α (TGF- α) is highly homologous with EGF and competes for EGF-receptor (EGF-R) binding (22). TGF- α stimulates growth of normal and tumorous tissues (22) and its expression by a variety of human carcinomas correlates with an unfavorable prognosis (23). TGF- α has been localized by immunohistochemistry in thyroid tissues and tumors (8,24). Driman and colleagues did not report any correlation of staining pattern with the pathophysiologic process (24); in contrast, Lemoine and co-workers reported negative staining in normal thyroid, positivity in thyroiditis, and variable staining in several tumor types (8) and an anaplastic thyroid carcinoma cell line secretes high levels of TGF- α (12). Although TGF- α mRNA levels were found to be consistently higher in papillary carcinomas than in nonneoplastic tissue of the same patient, there was a high degree of overlap in absolute levels of TGF- α mRNA in normal thyroid tissue and tumors (25). Consistent with these data, in our study there was no correlation between the degree of immunoreactivity and diagnosis or tumor grade. These results suggest that TGF- α plays a physiological role in thyroid growth but indicate that it is not likely to be useful as a tumor marker.

Abnormal expression of EGF-R has been implicated in carcinogenesis (22). Thyroid follicular cells are known to express EGF-R (26–31); although some authors have been unable to localize it by immunocytochemistry (32), the use of highly specific monoclonal antibodies has made this technique more reliable. One report indicated that the receptor has lower affinity in differentiated cancer than in normal or adenomatous tissues (21); in contrast, others have reported higher binding and expression by hyperplastic tissues and differentiated follicular and papillary carcinomas, with the highest levels in more aggressive tumors, such as anaplastic carcinomas (12,25,29,33,34). We were unable to reproduce the findings of Lemoine et al. (8) who reported increased immunopositivity for EGF-R in thyroid neoplasia; those same investigators did not find EGF-R gene amplification or gross rearrangements by Southern blotting of thyroid tumors. Further work is required to determine the role of EGF-R related oncogenes, such as the *c-erb-B* oncogene that has been found in papillary cancer (35), in the pathogenesis of thyroid carcinoma.

Cultured thyroid cells secrete IGF-1, IGF-2, and a high-molecular-weight form of IGF-1 that may represent the growth factor bound to one of its binding proteins (36–38). Minuto et al. suggested that the stronger staining for IGF-1 in human thy-

roid carcinomas than in normal tissue indicates a role for IGF-1 overexpression in thyroid tumorigenesis (38,39). IGF receptors have been localized on thyroid cells by specific binding (36,37,39) and by immunocytochemistry (39) and IGF-1 stimulates thyroid cell division synergistically with TSH (37,40). IGF binding sites are reported to be similar in number and affinity in nontumorous and in nodular thyroid tissue (38). However, there is evidence for autocrine IGF-1 action in thyroid follicular cells (41) and in a rat medullary thyroid carcinoma cell line (42). As shown in Table 1, in our study normal and abnormal thyroid tissue contained IGF-1 immunoreactivity, but we were not able to find any relationship between the number of positive cells or intensity of staining and biological behavior. We were unable to localize IGF-R in these tissues; this may be due to artefacts of fixation or other technical problems. Further studies will be required to verify if the previous reports of high affinity IGF-binding sites are truly reflective of IGF-R expression.

The results of immunostaining for IGFBP-1 and -4 are inconsistent in thyroid. In contrast, IGFBP-2 and -3 were selectively localized to medullary carcinomas, indicating cell-type specific expression consistent with their known production by neuroendocrine cells (43,44). The function of the IGFBPs is not known; they could be important in increasing the half-life of IGFs, presenting IGFs to their receptors, or modulating the activity of IGF-1 by binding free IGF-1.

Members of the transforming growth factor- β (TGF- β) family affect growth and differentiation of cells by stimulating differentiation and inhibiting growth (1). TGF- β binds to thyroid follicular cells with high affinity (45); it inhibits growth by TSH-receptor down-regulation (45–47) but it stimulates iodine trapping independent of TSH (45). TGF- β may, therefore, be a physiologically relevant negative modulator of thyroid growth. However, data concerning TGF- β expression by thyroid cells are scant. TGF- β -like activity is secreted by the FRTL-5 cell line (45). As shown in Table 1, we detected variable expression of TGF- β in the human thyroid, but there was no correlation of its localization with thyroid tumor proliferative activity or differentiation.

Literature concerning the role of the angiogenic factor FGF in the thyroid is scant. There has been a report of an endothelial cell growth factor produced by cultured porcine thyroid cells (48) and a recent paper reported abundant expression of bFGF by a follicular carcinoma cell line (49). bFGF was reportedly localized by immunohistochemistry in thyroid follicular and parafollicular cells and its receptor was confined to the microvasculature (50). bFGF has been shown to have an autocrine effect on thyroid follicular epithelial cells (51). We could not detect significant immunohistochemical staining for bFGF in our series; only two tumors had focal positivity and no staining was found in normal thyroid. This difference from the previous report may be due to the use of different antibodies or techniques; our study utilized both a polyclonal antiserum and a highly specific monoclonal antibody whereas the previous study applied a different polyclonal antiserum (50). It is not surprising that bFGF would be difficult to detect by immunolocalization, since it lacks a signal peptide and likely rapidly diffuses from the cell rather than being stored in detectable quantities.

In our survey, NGF was variably expressed by all types of

thyroid lesions, however, we were unable to totally abolish the staining by absorption, therefore the specificity of this finding remains to be proven. Several NGF precursor forms have been immunoprecipitated from homogenates of rat thyroid (52), but further studies will be required to prove the expression of NGF by thyroid tissues. The putative receptor for NGF has also not been analyzed in thyroid. Interestingly, we detected intense NGF-R positivity around blood vessels in normal thyroid and around large vessels of thyroid lesions, but no staining was seen in the stroma of thyroid lesions. This pattern is consistent with a vascular innervation network that may be absent in the angiogenesis of thyroid neoplasms.

The B chain of platelet-derived growth factor (PDGF) is homologous to the transforming protein of simian sarcoma virus (53) and PDGF is thought to be an important protooncogene. Heldin et al. documented expression of PDGF-receptors in an anaplastic thyroid carcinoma cell line (54) and Matsuo et al. reported production of a novel PDGF-B-like protein by a human papillary carcinoma cell line (49). Thus far we have not been successful with immunohistochemical localization of PDGF.

This study indicates that growth factors and growth factor receptors are expressed by thyroid follicular cells and C-cells. EGF, TGF- α , IGF-1, and EGF-R are widely expressed in non-tumorous thyroid tissue and in a wide variety of thyroid proliferative lesions. The IGF-R is not detected in thyroid but IGF-BPs show a specific staining pattern with BP-2 and -3 localized only in medullary carcinomas. NGF and TGF- β are variably expressed; however, bFGF cannot be consistently detected by immunohistochemistry in thyroid tissue. NGF-R stains are positive around vessels in normal thyroid tissues; however, in all the other benign or malignant thyroid lesions no positivity was found except around large vessels.

Although we could not find a correlation between the intensity and number of cells staining for any of the growth factors studied in the different thyroid tissues, they are still likely to play a role in cell regulation and proliferation in these tissues. Their immunohistochemical profile does not correlate with the pathological condition. Further studies will clarify the regulation of growth factor expression in the normal thyroid and the role of these growth factors in the modulation of cell proliferation, differentiation, and hormone synthesis.

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