Transformation of Thyroid Epithelium Is Associated with Loss of c-kit Receptor

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ABSTRACT

The receptor for the stem cell factor encoded by the c-kit proto-oncogene is expressed by a number of epithelial cells including thyrocytes. Since malignant transformation may be associated with loss of this receptor (melanoma and breast cancer), we have analyzed its expression in benign (38 cases) and malignant (31 cases) thyroid lesions. While low levels of c-kit are expressed in normal thyroids and in 60% of benign lesions, the receptor is undetectable in 60 and 90% of the follicular and papillary carcinomas, respectively. Northern blot analysis from surgical specimens of carcinomas and from carcinoma cell lines has demonstrated a lack of specific c-kit transcripts. These findings indicate that the c-kit receptor may be involved in the growth control of thyroid epithelium and that this function may be lost following malignant transformation.

INTRODUCTION

The c-kit proto-oncogene is the cellular homologue of the v-kit oncogene derived from the acutely transforming feline retrovirus HZ4-FeSV (1). The gene, which encodes a tyrosine kinase receptor, bears high homology with the platelet-derived growth factor and CSF-1 receptors (2). Studies of the dominant white spotting (w) murine locus (3) have clarified that the receptor regulates the development of a number of ontogenetically unrelated cell lineages, i.e., hematopoietic cells, germ cells, and melanocytes. These pleiotropic activities are mediated by the binding to a Mr 28,000–30,000 glycoprotein ligand identified as the SCF (4).

The development of monoclonal antibodies to c-kit have recently allowed us to address the issue of the tissue and tumor specificity of this receptor. These studies have demonstrated that the c-kit product displays a tissue distribution broader than that documented by genetic and molecular biology studies. One unexpected finding has been the expression of c-kit by thyrocytes (5, 6).

Since neoplastic transformation has been shown to be associated with either positive or negative regulation of c-kit expression (5–7), we have searched a variety of benign and malignant lesions of the thyroid epithelium for possible anomalies in c-kit expression. The data expressed here demonstrated that c-kit, while frequently expressed in benign proliferative lesions, becomes undetectable in the papillary type of thyroid tumors, c-kit expression is lowered also in about 60% of the follicular type of thyroid carcinomas. These findings were confirmed by Northern blot analysis data that demonstrated a lack of specific c-kit transcripts in most of the human papillary and anaplastic carcinomas analyzed.

MATERIALS AND METHODS

Cell Lines, Tissues, and Immunohistochemical Studies. The long-term thyroid carcinoma cell lines were established as described: TPC-1 (8); WRO (9); N-pap, AR0 (10); FRO (11); B-C pap (12). The human leukemic cell line M-07e (13), expressing high levels of c-kit receptor, was kindly provided by Professor L. Pegoraro (University of Turin, Turin, Italy). The lymphoblastoid cell line WI-L2 was obtained from American Type Culture Collection (Bethesda, MD). All cell lines were maintained in RPMI 1640 (GIBCO, Paisley, United Kingdom) supplemented with heat inactivated 10% FCS containing 2 mM L-glutamine.

Normal and neoplastic thyroid specimens were obtained from patients free from therapy and undergoing surgical treatment at the Regina Elena Cancer Institute or at the Hôpital de l’Antiquaille (Lyon, France). Histological classification followed WHO recommendations (14). Upon removal, tissues were divided into two parts; one was processed for routine histopathological examination, and the other was snap frozen in liquid nitrogen and stored at −20°C or −80°C prior to use in immunohistochemical or RNA assays, respectively.

Four-μm cryostat sections were obtained and fixed in cold absolute acetone for 10 min. Sections were either immediately used as a substrate for indirect immunoperoxidase or stored at −20°C up to 6 months with no appreciable changes in reactivity. Sections were also stained with 1% toluidine blue for morphological evaluation. At least three, nonconsecutive sections of each biopsy were analyzed.

The murine mAb 1D29.3D6 to the extracellular domain of the c-kit was purchased from Boehringer Mannheim (Milan, Italy). The mAb immunoprecipitates a single component of Mr 135,000 ± 145,000. mAb DO24 to the extracellular domain of the MET/HGF product was produced as described (15). mAb W8000 to an epitope of the extracellular portion of the gp185HER-2 was obtained as reported (16). Rabbit antiseraum L111 against a peptide corresponding to the COOH-terminal 17-amino acid residues of the human p145 c-kit was kindly provided by Dr. A. Ullrich (Max Planck Institute, Martinsried, Germany).

The purified reagents were used at concentrations ranging from 10 to 50 μg/ml. Indirect immunoperoxidase staining was performed with commercially available reagents (Vectastain, Mountainview, CA). Slides were incubated overnight with mAb at 4°C in a moistened chamber. The enzymatic activity was developed using 3-amino-9-ethylcarbazole as chromogenic substrate for 8 min. Slices were then rinsed with PBS and counterstained with Mayer’s hematoxylin. Sections on which the incubation with the primary antibody was omitted were used as controls.

Western and Northern Blot Analysis. For Western blot analysis, surgical biopsies were immediately frozen in liquid nitrogen and pulverized using a Mikro Dismembrator (B-Braun) in the presence of liquid nitrogen. Powdered tissues were solubilized in boiling Laemmli buffer (17) containing the reducing agent β-mercaptoethanol. Four hundred μg of proteins were loaded on each lane. Western blot analysis was carried out as described by Towbin et al. (18). Bound antibodies were revealed with rabbit anti-mouse antibodies labeled with horseradish peroxidase according to the enhanced chemiluminescence method (ECL; Amersham).

Total RNA was isolated from different tissue samples according to Chomczynski and Sacchi (19) as modified by Puissant and Houdebine (20). Ten μg of each RNA were analyzed on formaldehyde agarose (1.2%) gels as described by Lehrach et al. (21). The RNA was transferred to nitrocellulose or nylon filters and hybridized to a radiolabeled EcoRI/Apal restriction fragment (1312 base pairs) isolated from cloned c-kit cDNA (2). Filters were washed and exposed to X-ray films at −70°C for 5 days using an intensifying screen (Cronex Lightning Plus).
RESULTS

Expression of c-kit Receptor in Normal Thyroid and in Benign and Malignant Thyroid Lesions. Staining of normal thyroid tissue with a mAb to the extracellular domain of c-kit demonstrated that the SCF receptor is expressed at low levels but with a high degree of homogeneity by thyrocytes, with a predominant basal distribution. The observation of a baso-lateral stain was much influenced by the section plane (Fig. 1, A and B). In 2 of 14 normal thyroid samples tested, no c-kit product could be observed. Whether this finding is related to a low sensitivity of the assay used was not further evaluated. Western blot analysis of extracts of normal thyroid tissue using polyclonal antiserum to the cytoplasmic domain of the receptor confirmed the immunohistochemical data. The c-kit product expressed by thyrocytes has a molecular weight of 135,000 (Fig. 2), slightly lower than that of the c-kit product expressed by a control hematopoietic cell line; this may reflect differences in phosphorylation and/or tissue-specific glycosylation, as already shown by others (22).

Immunohistochemical analysis of benign thyroid lesions (Table 1) demonstrates that the c-kit gene product is heterogeneously expressed in macro and microfollicular goiters, among which 60% of the cases were found positive. The latter lesions did not display histopathological features distinguishable from the cases where no c-kit expression could be detected. The receptor was expressed in 7 of 11 follicular adenomas. Also in the latter cases, no histological features could distinguish SCF receptor positive from negative specimens.

Staining of a representative number of thyroid carcinomas, which included 10 follicular and 21 papillary tumors (Table 1; Fig. 1, C and D), demonstrated that detectable levels of the c-kit receptor were found only in 1 papillary carcinoma and in 3 follicular tumors. Very weak staining was observed in the positive cases. A comparative analysis of the expression of the c-MET oncogene product, on the other hand, revealed that the HGF receptor was overexpressed in a minority of goiters and adenomas, in all follicular carcinomas, and in 17 of 21 papillary carcinomas tested. The gp185HER-2, known to be expressed by the thyroid epithelium (23), was homogeneously present at low levels in all cases studied, with no significant changes from the reactivity shown by normal follicular epithelium, except for one case in which the HER-2 gene product appeared to be overexpressed.

In order to ascertain whether the lack of the c-kit gene product in primary thyroid carcinomas is also maintained in metastatic foci, we examined the expression of the receptor in multiple metastases of four primary tumors. The results of this study (Table 2) have demonstrated that, in three papillary tumors, the lack of the receptor is maintained in the autologous metastases. In one case of follicular carcinoma, in which detectable levels of the receptor were expressed in the primary lesion, no c-kit product could be demonstrated in the corresponding metastases. At variance with the c-kit receptor, the product encoded by the met proto-oncogene could be detected in all lesions independently of their primary or metastatic nature.

Fig. 1. Immunohistochemical analysis of the expression of the c-kit gene product on 4-μm acetone-fixed cryostat sections by indirect avidin-biotin immunoperoxidase assay. The distribution pattern of the receptor appears predominantly along the epithelial surface abutting the basement membrane (A), although in some section planes a plasma membrane stain can be observed (B, arrow). No detectable levels of the receptor are expressed in a case of papillary (C) and of follicular carcinoma (D), as demonstrated by the lack of plasma membrane stain of tumor cells. (A–D, × 200).
MODULATION OF c-kit RECEPTOR IN THYROID TUMORS

Because differences in Southern blot patterns of c-kit DNA of normal thyroid and of transformed cells could not be detected (data

Table 2 Comparative analysis of the expression of c-kit and c-MET gene products in primary thyroid carcinomas and autologous concomitant metastases

<table>
<thead>
<tr>
<th>Case</th>
<th>Histotype</th>
<th>Lesions</th>
<th>Receptor expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>c-kit</td>
</tr>
<tr>
<td>MA</td>
<td>Papillary</td>
<td>Primary tumor</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 1</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 2</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 3</td>
<td>Neg</td>
</tr>
<tr>
<td>PA</td>
<td>Papillary</td>
<td>Primary tumor</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 1</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 2</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 3</td>
<td>Neg</td>
</tr>
<tr>
<td>IN</td>
<td>Follicular</td>
<td>Primary tumor</td>
<td>Var</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 1</td>
<td>Neg</td>
</tr>
<tr>
<td>SA</td>
<td>Papillary</td>
<td>Primary tumor</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 1</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 2</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met no. 3</td>
<td>Neg</td>
</tr>
</tbody>
</table>

* Var, staining of heterogeneous intensity; +/-, weak homogenous stain; +, homogenous stain. MET, metastases.

Northern blot analysis of c-kit mRNA was performed on a series of long term cell lines and of normal and neoplastic thyroid tissues. Fig. 3 shows a Northern analysis of six different thyroid tumor cell lines, obtained from one follicular, three papillary, and two anaplastic thyroid carcinomas, respectively. This study demonstrates that only in the case of the follicular tumor cell line is the expression of c-kit mRNA conserved (Fig. 3, Lane 3) compared to normal human thyroid tissue (Fig. 3, Lane 1). All other tumor-derived cell lines (Figs 3, Lanes 2, 4, 5, 7, and 8) did not show c-kit mRNA expression. However hybridization of the same filter with an action probe yielded a strong signal in all samples.

In Fig. 4, a Northern analysis of RNAs obtained from fresh thyroid tumors is shown; of six papillary carcinomas tested, only one showed the presence of the c-kit transcript. Of three follicular carcinomas, two scored positive and one negative for c-kit expression. In the case of the three anaplastic carcinomas, one showed the presence of low amounts of c-kit mRNA, whereas the other two were negative for c-kit expression.

Table 1 Comparative expression of c-kit, c-MET, and HER-2 protooncogene products in normal thyroid and in benign and malignant thyroid lesions

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Expression of the receptor encoded by:</th>
<th>c-kit</th>
<th>c-MET</th>
<th>HER-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal thyroid</td>
<td></td>
<td>12/14</td>
<td>0/14</td>
<td>12/14</td>
</tr>
<tr>
<td>Goiter (macro- microfollicular)</td>
<td></td>
<td>15/27</td>
<td>3/27</td>
<td>19/21</td>
</tr>
<tr>
<td>Adenoma</td>
<td></td>
<td>7/11</td>
<td>2/8</td>
<td>7/7</td>
</tr>
<tr>
<td>Follicular carcinoma</td>
<td></td>
<td>3/10</td>
<td>8/8</td>
<td>7/8</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td></td>
<td>1/21</td>
<td>17/21</td>
<td>13/14</td>
</tr>
</tbody>
</table>

* No. of position/no. tested. Expression was scored positive when a clear outline of the cell membrane was observed.
Fig. 4. Analysis of the expression of the c-kit gene in normal thyroid and tumoral thyroid tissues. Ten μg of total RNA for sample were size fractionated on a denaturing formaldehyde agarose gel, blotted onto Nylon filters (Hybond-N; Amersham), and probed with the c-kit and actin genes, as indicated. The sources of the RNAs were the following: Lane 1, normal thyroid; Lanes 2–6 and 10, papillary carcinomas; Lanes 7–9, follicular thyroid tissues. Ten μg of total RNA for sample were size fractionated on a denaturing formaldehyde agarose gel, blotted onto Nylon filters (Hybond-N; Amersham), and probed with the c-kit and actin genes, as indicated. The sources of the RNAs were the following: Lane 1, normal thyroid; Lanes 2–6 and 10, papillary carcinomas; Lanes 7–9, follicular thyroid tissues.

not shown), the lack of RNA transcripts in the latter is unlikely to be related to gross gene rearrangements.

DISCUSSION

The molecular events underlying the transformation of the thyroid epithelium are just beginning to elucidate. Activating mutations of RAS genes are frequently found in follicular adenomas (24), thus suggesting that deregulation of RAS activity may play a role in the early stages of thyroid cell transformation. Rearrangements of the RET and TRK (25, 26) oncogenes, which confer transforming activity to the mutated allele, have been detected in about 50% of tumors of the papillary histotype. This suggests that at least in this histological variety of thyroid carcinomas, molecular lesions of oncogenes encoding TKRs may be of pathogenetic relevance (27). This is supported by the recent report that also the c-MET protooncogene, which encodes the HGF receptor is overexpressed in more than 70% of the thyroid tumors of the papillary histotype (28).

Recent work has documented that c-kit, the receptor for SCF, is expressed in a number of nonhematopoietic cell lineages, including normal thyroid epithelium (5). In this study, we have confirmed and extended this observation by hybridizing Northern blots of RNAs obtained from normal thyroid glands with a c-kit cDNA probe as well as by immunoblotting extracts of thyroid tissue with a c-kit-specific antiserum. Therefore, we set out to investigate whether SCF receptor expression is modulated in the course of thyroid epithelium neoplastic transformation. While c-kit expression was preserved in a large fraction of goiters and benign adenomas, a very high percentage of primary papillary thyroid carcinomas and a considerable fraction of follicular carcinomas did not display detectable levels of the receptor. The absence of the c-kit gene product was demonstrated also in multiple autologous metastases from four patients bearing primary papillary carcinomas and from one patient bearing a follicular carcinoma, thus indicating that modulation of the receptor in the primary tumor is not dependent on the thyroid microenvironment but is intrinsic to the transformed phenotype. These results were confirmed by Northern blot analysis of RNAs obtained from both established tumor cell lines and thyroid cancer surgical biopsies.

In general, loss of c-kit expression was more frequent in anaplastic and papillary thyroid carcinomas than in follicular tumors. This is of interest since molecular lesions of the TRK and RET tyrosine kinase receptors occur frequently in papillary carcinomas but never in follicular thyroid tumors (26, 27).

The biological significance of loss of c-kit in thyroid malignancies is not clear. SCF is not mitogenic for primary cultures of thyrocytes either per se or in conjunction with thyroid-stimulating hormone, a result which would indicate that the SCF/c-kit pathway may control some aspects of the thyrocyte differentiated phenotype rather than cell division. This would agree with the apparently strong selection for loss of c-kit expression in neoplastic transformation of the thyroid epithelium. This negative selection is in stark contrast with the gain of function in genetic lesions of the TRK and RET tyrosine kinase receptor (TKR) and the overexpression of c-MET frequently found in thyroid carcinomas (28). Also in breast carcinomas, loss of c-kit expression (29) is often mirrored by overexpression of gp180HER-2 (30), a TKR implicated in cell transformation (31). The molecular pathology of breast and thyroid carcinomas fits into the conceptual framework that different TRK signaling pathways may elicit opposite biological effects in a given cell type. In this context, it is also not surprising that c-kit expression is retained in seminomas (32), gliomas (33), and small cell lung carcinomas (34), since tyrosine kinases such as v-fms (35, 36) or v-src (37, 38) may control mitogenesis or differentiation, depending on the cell type in which they are expressed.

It is clear that more work is needed to elucidate the biological role of the SCF/c-kit pathway in thyrocytes and the impact of c-kit loss during tumor progression. In light of our results, it will be especially interesting to study whether heterologous c-kit expression in thyroid carcinoma cell lines exerts dominant negative activity on the transformed phenotype.

Although the functional consequences of this modulation are presently unknown, c-kit function is likely to be relevant in regulating thyrocyte differentiation and survival, thus loss of the SCF receptor may be a necessary step toward tumorigenesis.

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