Block of c-myc Expression by Antisense Oligonucleotides Inhibits Proliferation of Human Thyroid Carcinoma Cell Lines

Janete Cerutti, Francesco Trappasso, Caterina Battaglia, Li Zhang, Maria Luisa Martelli, Roberta Visconti, Maria Teresa Berlingieri, James A. Fagin, Massimo Santoro, and Alfredo Fusco

INTRODUCTION

Elevated expression of MYC proteins can induce oncogenic transformation (1, 2) and apoptosis (3, 4) and can block cell differentiation (5, 6). The proteins belonging to the MYC protein family are localized in the nucleus (7–9) and can activate transcription when brought into contact with DNA by heterol-ogous DNA-binding domains (10–12). Recently, two proteins that form heterodimers with MYC proteins have been isolated: Max, a human protein; and Myn, its murine homologue (13, 14). The c-myc gene was found to be rearranged, amplified, and overexpressed in a wide variety of human cancers (15). It was also shown to be involved in the progression of various cancers (16, 17). In the thyroid systems, c-myc can cooperate with some viral oncoproteins in inducing the neoplastic phenotype in the rat thyroid cell line PC 13 (18). Furthermore, there is a positive correlation between elevated levels of c-myc expression and the stages of human thyroid neoplasia (19). However, the role of c-myc overexpression in the process of thyroid carcinogenesis is still unknown.

We have analyzed c-myc expression in 7 cell lines originating from different histotypes of human thyroid carcinomas and in 50 fresh thyroid carcinomas. Levels of c-myc were observed in all thyroid carcinoma cell lines; the highest increase was detected in the more malignant, undifferentiated cell lines. Interference with c-MYC protein synthesis by antisense oligonucleotides inhibited the growth of thyroid carcinoma cell lines.

MATERIALS AND METHODS

Cell Culture and Assay of the Transformed State. The human thyroid carcinoma cell lines studied were TPC-1 (20), WRO (21), NPA (22), ARO (22), FRO (22), NIM 1 (24), and B-CPAP (25). They were grown in DMEM containing 10% fetal bovine serum. HPCs were established as described (26). Tu-morigenicity of the cell lines was tested by injecting 2 × 10⁶ cells into athymic mice. Soft-agar colony assays were performed as described elsewhere (27).

RNA Isolation and Northern Blot Analysis. Thyroid tumors were obtained from the Laboratoire d’Histologie et de Cytologie, Centre Hospitalier (Lyon Sud, France). The tumor samples were frozen in liquid nitrogen and stored frozen until RNA was extracted. The procedures for total RNA extractions, Northern blots, and hybridizations have been described previ-ously (28). The c-myc probe used in this study was the 1.3-kb PstI-PstI fragment of the pRyc 7.4 plasmid, which is specific for the human myc gene (29). The probe for the max gene was obtained by PCR amplification using the max-specific primers (13) 5′-CCTGGGCGGTAGGAAATGACGATAAAC-3′ and 3′-GATGGAGCACGAGAAGTTG3′.

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The abbreviations used are: HPC, human primary culture; NFDM, nonfat dried milk; TBS, Tris-buffered saline.
3'-TGCGACGCTTTAGCTGCGCTCCA-5'. A mouse β-actin probe was used to ascertain the equal RNA loading (30).

**Protein Isolation and Western Immunoblot Analysis.**

The protein isolation procedure has been described elsewhere (31); 5 × 10⁶ cells were washed twice with PBS, then lysed in a buffer containing 10 mm sodium phosphate (pH 7.4), 0.1 m NaCl, 1% Triton X-100, 0.1% SDS, sodium deoxycholate, 1 mm phenylmethylsulfonyl fluoride, 10 mg aproatin/ml, 10 mg leupeptin/ml, and 10 mg pepstatin/ml. Extracts were clarified by sedimentation in a microcentrifuge at low speed for 5 min at 4°C. The sample of normal thyroid tissue was ground to a fine powder in a mortar with a pestle and sand. The powdered tissue was then resuspended in an equal volume of ice-cold protein extraction buffer. Thereafter, the proteins were extracted using the procedure used for the cell lines. The protein concentration of the supernatant was determined by the Bio-Rad protein microassay as specified by the manufacturer (Bio-Rad, Watford, England).

Western immunoblot analysis was performed according to a standard procedure (32). Briefly, protein fractionation was carried out by SDS-PAGE using 80 μg (or 40 μg) cell protein samples/lanes of a 10% polyacrylamide gel. Before loading, proteins were diluted in loading buffer containing 10% glycerol, 2% SDS, 62.5 mm Tris-HCl (pH 6.8), 5% β-mercaptoethanol, and 0.5% bromophenol blue. Gels were transferred to nitrocellulose in transfer buffer (390 mm glycine, 480 mm Tris, 0.37% SDS, and 20% methanol). The filters were incubated for 1 h at room temperature in 1% NFDM and TBS buffer [200 mm Tris-HCl (pH 7.9) and 1.5 m NaCl]. After washing, the filters were incubated for 1 h in TBS buffer with Tween 20 [200 mm Tris-HCl (pH 7.9), 1.5 m NaCl, and 0.5% Tween 20] plus 1% NFDM containing 10 μg/ml antibody c-myc (Ab-1; Oncogene Science, Inc., Uniondale, NY). After four washes, blots were incubated with antimouse antibodies conjugated with horseradish peroxidase diluted 1:1000 for 1 h in TBS buffer with Tween 20 plus 1% NFDM. Parallel gels were run to ascertain that equal amounts of protein in fact had been loaded by staining with Coomassie blue. Revelation was performed with Enhanced Chemiluminescence (Western blot detection kit; Amersham, Amersham Place, United Kingdom).

**Effects of Antisense c-myc on c-MYC Protein Expression and Cell Growth.**

The oligonucleotides used were identical or complementary to sequences at the beginning of the coding region of c-myc in exon 2. The sense oligomer used was 5'-ATGCCCCTCAACGTT-3'; the antisense oligomer was 3'-TACGGGGAGTTGCAA-5'; the missense oligomer was 3'-AAGCTTGAAGCGAT-5' (33). The missense oligonucleotide was complementary to only a few reading frames of human T-cell lymphotropic virus, type I, and a portion of the neural cell adhesion molecule (34). Phosphorothioate oligonucleotides were synthesized by a modification of the H-phosphonate procedure (35), purified by ion-exchange chromatography, and ethanol precipitated.

To analyze the capability of the antisense oligonucleotides to block the abundance of the c-MYC protein, the cells were treated with 4 μm oligonucleotide 24 h after cell plating. Proteins were extracted 48 and 72 h after oligonucleotide treatment, and the levels of the c-MYC protein were measured by Western blot (see above).

**Southern Blot Analysis.** The Southern blot analysis was performed according to a standard procedure (28).

**DNA Synthesis Assay.** To evaluate the effects of the oligonucleotides on the cell growth rate, 10⁴ cells were plated, and 15 h later, they were treated with sense, missense, and antisense oligonucleotides. After 24 and 72 h, DNA synthesis was measured by incorporation of [³H]thymidine (2 μCi/ml, 40 Ci/mmol; Amersham) into trichloroacetic acid-insoluble material as described elsewhere (36). The results are expressed as percentage of inhibition of thymidine incorporation in comparison with the untreated cells. Each value is the mean of at least three experiments in duplicate. Thymidine uptake values were analyzed by one-way ANOVA. Differences were considered statistically significant at P < 0.05.

**Cellular Uptake of Oligonucleotide.** Five × 10⁵ HPC, FRO, and ARO cells in 100 μl culture medium with heat-inactivated fetal bovine serum were exposed to 5 μM 5' end-labeled oligonucleotide for 16 h at 37°C. Fetal bovine serum was heated to 65°C for 30 min to inactivate DNases. The cells were washed twice, and the supernatants were saved. The percentage of uptake by the cells was the number of counts in the cell pellet/total number of counts in the supernatant. The results reported are the average of two duplicate experiments.

**RESULTS**

**c-myc Expression in Human Thyroid Carcinoma Cell Lines.** Seven thyroid carcinoma cell lines (four derived from papillary carcinomas, two from anaplastic carcinomas, and 1 from a follicular carcinoma) were analyzed for c-myc expression by Northern blot. The origin of each cell line and their tumorigenic properties are listed in Table 1. Levels of myc-specific transcripts were higher in the thyroid cell lines than in normal human thyroid primary culture cells (Fig. 1). There was a 5-fold increase in NIM1 and B-CPAP cells, a 10-fold increase in WRO cell and TPC-1 carcinoma cell lines, and a very high increase (between 50- and 100-fold) in the ARO and FRO cell lines that originated from anaplastic carcinomas. It is noteworthy that the ARO and FRO cell lines induced tumors in athymic mice and had the highest colony-forming efficiency in agar among the cell lines used in this study (Table 1). Because the myc gene product is known to form heterodimers with the

<p>| Table 1 Expression of the neoplastic phenotype of human thyroid carcinoma cell lines |
|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Carcinoma histotype</th>
<th>Colony-forming efficiency in agar (%)</th>
<th>Tumorigenicity in athymic mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC-1</td>
<td>Papillary</td>
<td>0</td>
<td>NO</td>
</tr>
<tr>
<td>WRO</td>
<td>Follicular</td>
<td>8</td>
<td>YES</td>
</tr>
<tr>
<td>NPA</td>
<td>Papillary</td>
<td>18</td>
<td>YES</td>
</tr>
<tr>
<td>NIM 1</td>
<td>Papillary</td>
<td>0</td>
<td>YES</td>
</tr>
<tr>
<td>B-CPAP</td>
<td>Papillary</td>
<td>0</td>
<td>NO</td>
</tr>
<tr>
<td>ARO</td>
<td>Anaplastic</td>
<td>70</td>
<td>YES</td>
</tr>
<tr>
<td>FRO</td>
<td>Anaplastic</td>
<td>38</td>
<td>YES</td>
</tr>
</tbody>
</table>

* Small tumors appeared after about 2 months. 
* Large tumors appeared after 2 weeks.
product of the max gene, we also analyzed max gene expression in the same cell lines. There was no difference in the abundance of c-max mRNA between tumor cell lines and normal thyroid (Fig. 1).

To investigate whether the increased c-myc-specific mRNA expression was also associated with an increased level of the MYC protein in the thyroid carcinoma cell lines, we analyzed the MYC protein levels in six cell lines (ARO, FRO, B-CPAP, NPA, WRO, and NIM1) in HPCs and normal thyroid by Western blot. The levels of the MYC protein were higher in all six cell lines, particularly the ARO and FRO cell lines (see Fig. 2, Lanes 2 and 3) than in normal thyroid tissue or primary culture cells (Fig. 2). A parallel gel stained by Coomassie blue confirmed that the amounts of proteins loaded were equal (data not shown).

The c-myc gene was amplified in the ARO and FRO cells (5-6- and 3-4-fold, respectively) compared with normal thyroid cells (Fig. 3). The same Southern blot was hybridized with a β-actin probe, and there was no difference among the same DNA.

c-myc Proto-oncogene Expression in Thyroid Tumors.

We analyzed 44 human thyroid neoplasias of different histological types (5 adenomas, 25 papillary carcinomas, 8 follicular carcinomas, and 6 anaplastic carcinomas) and 6 nodular goiters for c-myc proto-oncogene expression (see Table 2). No significant increase in expression of c-myc was observed in adenomas or in follicular carcinomas; expression was increased slightly in 4 of 25 papillary carcinomas. Five of the 6 anaplastic carcinomas expressed very high amounts of c-myc mRNA. Some representative data are shown in Fig. 4. In four papillary carcinomas, the levels of c-myc expression are comparable with those observed in normal thyroid, whereas 2 anaplastic carcinomas showed almost 50-fold increases in c-myc expression. The RNAs were normalized according to the levels of β-actin expression.

Effect of Blockage of MYC Protein Synthesis on Growth of Thyroid Carcinoma Cell Lines. Next, we determined the role of the enhanced c-myc expression in the process of cell transformation and growth by blocking the synthesis of
Table 2  Expression of c-myc proto-oncogene in human thyroid neoplasias

<table>
<thead>
<tr>
<th>Histological type of thyroid specimens</th>
<th>No. of patients with elevated level of c-myc mRNA/no. of patients analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillary carcinomas</td>
<td>4/25</td>
</tr>
<tr>
<td>Follicular carcinomas</td>
<td>1/8</td>
</tr>
<tr>
<td>Anaplastic carcinomas</td>
<td>5/6</td>
</tr>
<tr>
<td>Adenomas</td>
<td>0/5</td>
</tr>
<tr>
<td>Goiters</td>
<td>0/6</td>
</tr>
</tbody>
</table>

*a c-myc RNA levels were considered elevated when they were at least 3-fold higher versus the level found in normal thyroid. The expression of the β-actin gene was used to normalize the levels of RNA loaded on the gel.*

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**Fig. 4** Analysis of c-myc expression in normal and neoplastic thyroid tissues. Ten μg total RNA for each sample were size fractionated on a denaturing formaldehyde agarose gel, blotted onto nylon filters (Hybond-N; Amersham), and probed with either c-myc or β-actin cDNA, as indicated. RNA was extracted from the following sources: Lane 1, normal thyroid; Lanes 2, 7, 9, and 10, anaplastic carcinomas; Lanes 3, 4, 5, and 6, papillary carcinomas; and Lane 8, a follicular carcinoma. Actin was used as an internal control for uniform RNA loading.

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the c-MYC protein with an antisense oligonucleotide against the translation initiation region of the c-myc mRNA located at the beginning of exon 2. The efficiency of this antisense oligonucleotide to block MYC protein synthesis was evaluated by treating the ARO cells with the oligonucleotides and measuring MYC protein levels by Western blot 48 and 72 h later. MYC protein levels were significantly (at least 5-fold) lower in the ARO cells treated with the myc antisense-specific oligonucleotide (Fig. 5A, Lanes 2 and 3) than in ARO cells (Lane 1) or in cells treated with a sense myc-specific oligonucleotide (Lane 4). To ascertain that equal amounts of protein had been loaded on the well, parallel gels were run and stained with Coomassie blue (Fig. 5B). Almost identical results were obtained with the FRO cell line (data not shown).

The effects of the antisense myc oligonucleotide on the growth of the ARO, FRO, and NPA cells were analyzed by evaluating the DNA synthesis measured as [³H]thymidine incorporation after exposure to the oligonucleotides for 24 and 72 h. ARO and FRO thyroid carcinoma cell lines were used for these experiments, because they express high levels of c-myc-specific mRNA and MYC protein (Table 1).

Treatment with antisense myc oligonucleotides reduced the thymidine incorporation by the NPA, ARO, and FRO cells significantly (Table 3). The most striking inhibitory effect (about 80% compared with the untreated cells after 72 h) was exerted on the FRO cell line, which also had the highest expression of the MYC protein. These effects were specific, because no or only a very slight effect (inhibition of thymidine incorporation was not more than 10%) was detected in the experiments with sense or missense oligonucleotides. Moreover, inhibition of thymidine incorporation decreased with increasing concentrations of antisense c-myc oligonucleotides (data not shown). c-myc phosphorothioate antisense oligonucleotides exerted no inhibitory effect on a human thyroid primary culture (Table 3). These differences were not due to a different uptake of the antisense oligonucleotides by normal and neoplastic cells, because both cell types showed comparable uptake of the oligonucleotide (Table 4).

Sense and antisense oligonucleotides did not exert a significant toxic effect (<5%), measured by counting trypan blue-
sense and missense oligonucleotides, then \[^{3}H\]thymidine incorporation was assayed after 24 and 72 h as described in “Materials and Methods.”

Each value is the mean ± SE of three independent experiments performed in duplicate.

\(^{c}\) P < 0.05 versus the respective values obtained using the c-myc sense and missense oligonucleotides.

\(^{d}\) P < 0.01 versus the respective values obtained using the c-myc sense and missense oligonucleotides.

**Table 3** Inhibition of \[^{3}H\]thymidine incorporation by c-myc antisense, sense, and missense oligonucleotides

<table>
<thead>
<tr>
<th>Cell type</th>
<th>c-myc oligomer treatment</th>
<th>% inhibition after 24 h (^{a})</th>
<th>% inhibition after 72 h (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>Sense</td>
<td>11 ± 3</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>HPC</td>
<td>Antisense</td>
<td>12 ± 4</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>ARO</td>
<td>Sense</td>
<td>8 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>ARO</td>
<td>Antisense</td>
<td>35 ± 5(^{a})</td>
<td>50 ± 6(^{a})</td>
</tr>
<tr>
<td>ARO</td>
<td>Missense</td>
<td>6 ± 3</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>FRO</td>
<td>Sense</td>
<td>6 ± 2</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>FRO</td>
<td>Antisense</td>
<td>65 ± 6(^{a})</td>
<td>80 ± 10(^{a})</td>
</tr>
<tr>
<td>FRO</td>
<td>Missense</td>
<td>6 ± 2</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>NPA</td>
<td>Sense</td>
<td>9 ± 2</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>NPA</td>
<td>Antisense</td>
<td>20 ± 5(^{a})</td>
<td>34 ± 6(^{a})</td>
</tr>
<tr>
<td>NPA</td>
<td>Missense</td>
<td>10 ± 3</td>
<td>12 ± 4</td>
</tr>
</tbody>
</table>

\(^{a}\) Subconfluent cells were treated with c-myc sense, antisense, and missense oligonucleotides, then \[^{3}H\]thymidine incorporation was assayed after 24 and 72 h as described in “Materials and Methods.”

**Table 4** Cellular uptake of labeled myc antisense oligonucleotide

<table>
<thead>
<tr>
<th>Cell type</th>
<th>% oligonucleotide uptake (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>0.80 ± 0.20</td>
</tr>
<tr>
<td>ARO</td>
<td>0.70 ± 0.23</td>
</tr>
<tr>
<td>FRO</td>
<td>0.93 ± 0.27</td>
</tr>
<tr>
<td>NPA</td>
<td>0.85 ± 0.15</td>
</tr>
</tbody>
</table>

\(^{a}\) The percentage of the oligonucleotide uptake was calculated by the number of counts in the cell pellet/total number of counts in the culture medium supernatant. Each value is the mean ± SE of three independent experiments performed in duplicate.

Table 4 Cellular uptake of labeled myc antisense oligonucleotide

It is well known that high expression of the c-myc proto-oncogene correlates with an undifferentiated phenotype. The expression of c-myc is down-modulated rapidly on induction of differentiation pathways in mouse erythroblastoma cells, human promyelocytic HL-60, monoblastic U-937, and proerythroid K-562 cells, murine primary keratinocytes, and F19 embryonal carcinoma cells (37). Moreover, repression of c-myc expression by antisense c-myc oligodeoxyribonucleotides induces differentiation of HL60 cells (33). Thyroid neoplasias are a useful model for studying whether c-myc expression is related to the expression of an undifferentiated phenotype. In fact, they include a broad spectrum of tumors with different phenotypic characteristics and different biological and clinical behavior: from the benign colloid adenomas through the slowly progressive, differentiated papillary and follicular carcinomas to the fatal anaplastic carcinomas (38).

We found higher expression of the c-myc proto-oncogene in seven human thyroid carcinoma cell lines than in normal thyroid cells or in normal thyroid tissue. This expression was much higher in the cell lines that expressed a higher degree of malignancy. Also, in fresh human tumors, c-myc overexpression was correlated closely with the anaplastic histotype. In contrast, c-myc expression was not enhanced in papillary and follicular carcinomas. The discrepancy between these data and those obtained from the carcinoma cell lines may be due to contamination of neoplastic tissue by normal thyroid tissue and tumor stroma and to the possibility that expression of c-myc transcripts might increase during their passages in culture. Moreover, the thyroid carcinoma cell lines were probably established from the most malignant thyroid carcinomas. Immunohistochemical analysis of paraffin blocks may reveal an increased level of MYC protein in papillary and follicular carcinomas, as has been demonstrated in 10 of 19 papillary thyroid carcinomas (39).

If this nuclear proto-oncogene represents the final pathway of the regulation of cell proliferation and differentiation, inhibition of nuclear oncogene expression should influence these processes. The results obtained in this study indicate clearly that c-myc overexpression is a very important event in the growth regulation of thyroid carcinoma cell lines, because c-myc antisense oligonucleotides inhibited the proliferation of carcinoma thyroid cell lines significantly. Moreover, the introduction of a dominant negative myc reduces the doubling time of the FRO cell line. Similarly, treatment of the CACO 320 cell line with c-myc antisense oligonucleotides inhibits their growth in semisolid medium (34). All these results indicate that the increase in the expression of the product of c-myc is an important event in the process of transformation of thyroid cells.

A number of mechanisms can be envisaged to explain the possible role of c-myc in thyroid cell transformation. It has been demonstrated that the protein coded for by the Rb gene and the c-MYC and N-MYC proteins can bind in vitro (40). Although such an association has not been demonstrated in vivo, the coinjection of c-myc and Rb suppresses the ability of Rb to arrest cell growth (40). Moreover, a dominant negative myc can block transformation by ABL oncogenes, indicating that myc overexpression is essential for the process of cell transformation induced by the v-ABL oncogene (41). Furthermore, the depletion of the c-MYC protein with specific antisense sequences reverses the transformed phenotype in ras oncogene-transformed NIH 3T3 cells, suggesting that a certain minimum level of c-myc is required to maintain ras transformation in NIH 3T3 cells (42). Indeed, our group has demonstrated that the myc oncogene cooperates with polyoma middle T and v-ras onco-
genes in the neoplastic transformation of rat thyroid cells (43, 44). Analogously, the overexpression of the c-myc gene could be essential for the human thyroid cell transformation induced by some oncogenes activated in thyroid neoplasias, including the ras genes detected with high frequency in the follicular histotype of human thyroid carcinomas (45–47) and the RET/PTC oncogene, found activated in about 20% of the thyroid papillary carcinomas (48). The activation of ras and RET/PTC must be considered an early event in the process of thyroid carcinogenesis, because both were detected in benign adenomas (49) and in occult thyroid papillary carcinomas (50), whereas c-myc gene overexpression might be involved in the progression stage of thyroid carcinogenesis.

The inhibition of the proliferation of human thyroid carcinoma cells lines by myc-specific antisense oligonucleotides indicates a role of c-myc overexpression in the process of human thyroid carcinogenesis strongly.
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