Overexpression of fibroblast growth factor receptor 3 in a human thyroid carcinoma cell line results in overgrowth of the confluent cultures

Hiroyuki Onose¹, Naoya Emoto¹, Hitoshi Sugihara¹, Kazuo Shimizu² and Ichiji Wakabayashi¹

¹Department of Medicine and ²Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603 Japan

(Correspondence should be addressed to N Emoto, Department of Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan)

Abstract

Recent reports indicate that a gain-of-function mutation in fibroblast growth factor receptor 3 (FGFR-3) inhibits cell growth in the cartilaginous growth plates. These results suggest that FGFR-3 may be the receptor transducing growth inhibitory signals. Using reverse transcription-PCR we examined seven papillary thyroid carcinomas to determine FGFR-3 expression. Six out of the seven papillary carcinomas expressed FGFR-3. To clarify the role of FGFR-3 in thyroid carcinoma, FGFR-3 was overexpressed in an established human papillary thyroid carcinoma cell line. High levels of FGFR-3 protein were identified in cells stably transfected with the vector containing FGFR-3 cDNA. The specific binding of ¹²⁵I-FGF-2 of these cells was threefold higher than that of control cells. Growth rates of cells overexpressing FGFR-3 were similar to those of control cells. However, cells overexpressing FGFR-3 continued to grow beyond the density at which control cells stopped proliferating. These results suggest that FGFR-3 in thyroid carcinoma is not involved strongly in the cell proliferation mechanism but may contribute to the malignant extension of some of the carcinomas by modifying cell contact signaling.

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Introduction

Fibroblast growth factor-2 (FGF-2), which promotes angiogenesis and stimulates thyroid cell growth *in vitro*, has been isolated from thyroids (1–5). Several reports have indicated that FGF-2 is increased in human papillary thyroid carcinoma (6–8). Recently, we found that intra-tissue extracellular matrix-free FGF-2 is also increased in human papillary thyroid carcinoma and Graves' disease-affected thyroid (9).

Expression of fibroblast growth factor receptors (FGFR) is required for the response of cells to FGF-2. Four human genes that encode FGFRs (FGFR-1, -2, -3 and -4) have been identified (10, 11). They have similar structures, consisting of three immunoglobulin (Ig)-like domains in the extracellular portion and two tyrosine kinase domains in the intracellular portion. Shingu *et al.* indicated that FGFR-1 was detected by immunohistochemical methods in most papillary thyroid carcinomas (12), and Thompson *et al.* showed that FGFR-1 was elevated in thyroid hyperplasia (13).

In the present study, we found that papillary thyroid carcinoma expressed FGFR-3. Recent reports indicate that a gain-of-function mutation in FGFR-3 inhibits cell growth in the cartilaginous growth plates and results in chondrodysplasia (14–19). These results suggest that

FGFR-3 may be the receptor transducing growth inhibitory signals. To examine the role of FGFR-3 in thyroid carcinoma, we overexpressed FGFR-3 in an established papillary thyroid carcinoma cell line (20) and investigated its effect on cell growth.

Materials and methods

Materials

Bovine calf serum was obtained from Nakarai Company (Kyoto, Japan). Ham's F-12 medium was purchased from Flow Laboratories (McLean, VA, USA), and the multiwell tissue culture plates were from Costar (Cambridge, MA, USA). Recombinant human FGF-2 was kindly supplied by Dr L Cousens (Chiron Corporation, Emeryville, CA, USA). Bolton-Hunter labeled human recombinant basic FGF (FGF-2) (¹²⁵I-bFGF; 1200–1300 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA).

Human thyroid tissues and tumors

Seven papillary thyroid carcinomas and adjacent normal thyroid tissues were obtained at thyroidectomy.

¹²⁵I-FGF-2 binding to TC cells was examined as described previously (5). Briefly, confluent cultures of TC cells in 12-well culture plates were washed with PBS, and the medium was changed to F-12 medium containing 0.15% gelatin and 25 mmol/l HEPES, pH 7.4. ¹²⁵I-FGF-2 (0.5–1.0 × 10⁵ c.p.m./ml) with or without unlabeled FGF-2 was added to the culture, and the cells were incubated for three hours at 4 °C. At the end of the incubation, after the 2.0 mol/l NaCl wash (pH

Figure 2 Southern hybridization analysis (lower panel) of RT-PCR products (upper panel: ethidium bromide staining) of FGFR-3 mRNA from human thyroids. RNA was extracted from normal human thyroid (lane 1), and human papillary thyroid carcinoma (lanes 2–8) and TC cells (lane 9), as described in the text.

7.4), the cells were solubilized with 1.0 mol/l NaOH, and an aliquot of the solution was used to determine radioactivity. We estimated the nonspecific high-affinity binding of ^{125}I -FGF-2 by adding 1.0 $\mu\text{g/ml}$ unlabeled FGF-2 to parallel cultures that were incubated under the same conditions, and we subtracted this value from the binding values obtained in all the high-affinity binding studies.

Results

Figure 2 shows the expression of FGFR-3 in papillary thyroid carcinoma and normal thyroid. Normal thyroid and six out of the seven papillary carcinomas expressed FGFR-3.

To investigate the role of FGFR-3, we overexpressed FGFR-3 in an established papillary thyroid carcinoma cell line (TC). Wild type TC cells expressed FGFR-1 and -2 mRNA (data not shown) in addition to FGFR-3 (Fig. 2). FGF-2 stimulated wild type TC cell growth in three days of culture (Fig. 3). Figure 4 shows the Western blotting of FGFR-3 extracted from TC cells stably transfected with the FGFR-3 cDNA or control

vector. A 12-kDa FGFR-3 band was barely detectable in extracts from control cells transfected with the vector (clones TCpc77-1, -2, -3, -4, and -5), but markedly higher amounts of the FGFR-3 band were observed in extracts from TC cells transfected with FGFR-3 (clones TCR377-2 and TCR377-3). One clone transfected with FGFR-3 (TCR377-1) did not show high levels of FGFR-3 expression. ^{125}I -FGF-2 binding to clones TCR377-2 and -3 was threefold higher than that to the control cells and TCR377-1 cells (Fig. 5). These results indicate that clones TCR377-2 and TCR377-3 overexpressed FGFR-3 on their cell surfaces.

Next, we examined the growth rate of TC cells stably transfected with FGFR-3 or control vector. In the presence of FGF-2 (10 ng/ml), the growth rates of clones TCR377-2 and TCR377-3 were not different from the growth rates of the control cells (TCpc77-1, -2, and -5). However, cells overexpressing FGFR-3 continued to grow beyond the density at which control cells stopped proliferating. At 15 days of culture, the cells reached a $75 \pm 16\%$ (mean $\pm$ s.e.) greater cell density ($P < 0.01$) than control cells (Fig. 6). After that, we could not estimate cell number precisely because of increased floating cells. In the absence of FGF-2, the growth rates and final cell density of TC cells overexpressing FGFR-3 were not significantly different from those of the control cells (data not shown). Similar results were obtained with two different clones (TCR377-7 and TCR327-6) overexpressing FGFR-3.

Figure 3 Effect of FGF-2 on TC cell growth. TC cells were cultured with the indicated concentrations of FGF-2 for three days. The cell number was determined as described in the text. Values are means $\pm$ s.d. of triplicate wells from one of three experiments.

Discussion

In the present study, we observed that normal thyroids and most papillary thyroid carcinomas expressed FGFR-3. Shingu *et al.* (12) detected FGFR-1 in papillary thyroid carcinomas immunohistochemically. Although FGF-2 stimulates thyroid cell growth *in vitro*, it is not known whether the FGF-2 cell proliferation signal is transduced by FGFR-1, -3 or both. On the other hand, recent reports of gain-of-function mutations in FGFR-3 suggest that FGFR-3 could be a growth inhibitory receptor (17, 19). We overexpressed FGFR-3 in an established

Figure 4 Western blotting of FGFR-3. The solubilized materials from TC cells transfected with control vector (clones TCpc77-1, -2, -3, -4, and -5; lanes 1-5) or vector containing FGFR-3 cDNA (clones TCR377-2 and -3; lanes 6 and 7) were subjected to SDS-PAGE and immunoblotted with anti-FGFR-3 antibody as described in the text.

human papillary thyroid carcinoma cell line (TC cells) to investigate the pathophysiological relevance of FGFR-3 expression in thyroid carcinoma.

TC cells are derived from human papillary cancer cells and synthesize insulin-like growth factor (IGF)-I, which stimulates growth by an autocrine mechanism (20). TC cell growth is also stimulated by FGF-2 (Fig. 3). This indicates that TC cells have the FGF-2 response system and are a good model system for investigating the role of FGFRs. As shown in Figs 4 and 5, overexpression of FGFR-3 in TC cells is markedly higher than in control cells, and it is possible that overexpression of FGFR-3 drastically changes the balance in expression of FGFRs. Thus, we examined the role of FGFR-3 in thyroid carcinoma growth in the present study.

Figure 5 The binding of ^{125}I -FGF-2 to TC cells. TC cells transfected with control vector (clones TCpc77-1, -2, -3, -4 and -5) or vector containing FGFR-3 cDNA (clones TCR377-1, -2, and -3) were incubated with ^{125}I -FGF-2 for three hours at 4 °C. The high-affinity binding of ^{125}I -FGF-2 was determined as described in the text.

Overexpression of FGFR-3 in TC cells had no effect on the growth rate of the cells. Naski *et al.* (18) showed in a pro B cell line that normal FGFR-3 expression is weak and less efficient than FGFR-1 in the mitogenic response to FGF, although activation of FGFR-3 promotes cell proliferation. On the other hand, Su *et al.* (17) indicated that the rate of DNA synthesis or colony-formation efficiency in 293T cells was reduced significantly by expression of an FGFR-3 gain-of-function mutant, suggesting FGFR-3 is an inhibitory receptor. These results suggest that the function of FGFR-3 is diverse in various tissues. Although it is possible that other FGFRs may affect the result, it seems reasonable to conclude that FGFR-3 in thyroid carcinoma is not involved strongly in the cell proliferation mechanism.

We found that overexpression of normal FGFR-3 in TC cells caused overgrowth of the confluent cultures.

Figure 6 Comparison of the growth curves of TC cells over-expressing FGFR-3 (clones TCR377-2 and -3) or TC cells transfected with control vector (clones TCpc77-1, -2, and -5). The cells were incubated in the medium containing 5% calf serum with 10 ng/ml FGF-2. The medium was changed and the treatment was renewed every other day. $P < 0.01$.

This effect must be due to overexpression of FGFR-3 stimulated by FGF-2, since it was not observed in the absence of FGF-2 or in the control cells. This suggests that FGFR-3 may be involved in contact inhibition. Because we examined one particular cell line in the present study, further studies will be necessary. However, this result suggests at least that FGFR-3 may contribute to the malignant extension of some papillary thyroid carcinomas by reducing the contact inhibition. The precise nature of the contact inhibition is not clear at this time. Cell contact-dependent signaling includes juxtacrine peptides, adhesion molecules and cell junction proteins (25). The effect of FGFR-3 overexpression on these factors is now under investigation in our laboratory.

In summary, we found FGFR-3 expression in thyroid carcinoma and normal thyroid. Overexpression of FGFR-3 in TC cells suggests that FGFR-3 may promote the malignant proliferation of thyroid carcinomas by altering contact inhibition. However, it is not clear whether the balance of FGFR-1 and -2 has any pathophysiological relevance in the growth of thyroid carcinoma. To address this question, experiments with combined overexpression of FGFRs in TC cells are now in progress in our laboratory.

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