行政院國家科學委員會專題研究計畫 成果報告

甲狀腺刺激素和上皮生長因子在甲狀腺癌細胞株上對於淋巴增生因子 血管內皮生長因子 作用之研究

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Introduction

Numerous investigations during the last decade have established the important role of VEGF as a key regulator of angiogenesis. Lymph node dissemination is a major prognostic factor in most human cancers. However, the molecular mechanisms underlying lymph node metastasis are poorly understood. More previously, several additional members of the VEGF gene family have been identified, including placenta growth factor, VEGF-B, VEGF-C, VEGF-D, and VEGF-E. VEGF-C and VEGF-D have been shown to stimulate the lymphangiogenesis.

Many clinical investigations have documented a positive correlation between VEGF-C levels in primary tumors and the presence of lymph node metastases. In cervical cancer and gastric cancer, there have been positive correlations between the VEGF-C expression in the primary tumors and a poorer prognosis. However, other studies in colorectal and pancreatic cancers failed to identify any correlation. Different thyroid cancers have a different propensity for lymph node metastasis. For example, papillary thyroid cancer tends to metastasize to regional lymph nodes, whereas follicular thyroid cancer usually metastasizes by a hematogenous rather than by a lymphatic route. The report from Fellmer et al. showed a significantly higher VEGF-C gene expression in papillary than in follicular thyroid cancers. Other investigations have previously documented a significant correlation between VEGF-C expression in primary thyroid cancer tissues and the occurrence of lymph node metastases. In previous investigation at UCSF, we also found that papillary thyroid cancers had a higher VEGF-C expression than other thyroid malignancies. Paired comparison of VEGF-C expression between thyroid cancers and normal thyroid tissues from the same patients showed a significant increase of VEGF-C expression in papillary thyroid cancer and a significant decrease of VEGF-C expression in medullary thyroid cancer. In contrast, there was no significant difference of VEGF-C expression between cancer and normal tissues in other types of thyroid cancer.

VEGF-C mRNA expression is upregulated by serum and various growth factors, such as platelet-derived growth factor, epidermal growth factor, transforming growth factor-β and the tumor promoter phorbol myristate 12,13-acetate. Thyroid stimulating hormone (TSH) is documented to stimulate tumor growth and metastasis in thyroid cancers. To suppress TSH is thought to be able to control thyroid tumor growth and metastasis. Clinically, thyroid suppression therapies using thyroid hormone have been applied to the treatment of benign thyroid nodules and control of thyroid malignancy postoperatively. Soh et al. have reported TSH may promote growth in some thyroid cancers by stimulating VEGF secretion and angiogenesis. In vivo study from Viglietto et al. also demonstrated that in the thyroid gland of thiouracil-fed rats, increased mRNA and protein expression of VEGF and Flk-1/KDR occurred subsequent to the rise in the serum TSH levels and in parallel with thyroid capillary proliferation. However, the effect of TSH on lymphangiogenesis through VEGF-C and/or VEGF-D in thyroid cancers has not been reported yet.

Thyroid cancers are known to arise and proliferate in an epidermal growth factor (EGF)-rich environment and possess EGF receptors. Overexpression of epidermal growth factor receptor and related erb-B receptor tyrosine kinase has been demonstrated in many human tumors. The presence of erb-B family receptors has been demonstrated in normal and neoplastic thyroid tissue, as well as thyroid cancer cell line. Stimulation of thyroid cancer cell lines with epidermal growth factor was shown to enhance in vitro metastasis. However, the correlation of epidermal growth factor and lymphangiogenic factors, VEGF-C and VEGF-D, in thyroid cancer has not been clearly clarified. In this study, we try to clarify the
role of TSH and epidermal growth factor on the expression of lymphangiogenic factors, VEGF-C and VEGF-D, in thyroid cancer cell lines.

**Material and Methods**

**Cell line and TSH/EGF treatment**

In this study, we used 2 thyroid cancer cell lines—TPC-1 and ONCO-DG1. The thyroid cancer cell lines, TPC-1, are maintained in regular media (Dulbecco’s modified Eagle’s media (DMEM)/F12 supplemented with 10% fetal calf serum (FCS), insulin, glutamine, TSH, and antibiotics) in standard incubating conditions (5% CO₂, 95% humidity, 37°C). 500,000 TPC-1 cells are seeded in Petri dishes containing 10 ml regular media for 24 hours. To exclude any TSH effects, cells then are maintained for 24 hours in TSH-free H5 medium (DMEM/F12 supplemented with 10% FCS, insulin, human transferrine, somatostatin, glycyl-histidyl-1-lysine acetate, hydrocortisone, and glutamine). Thereafter, the cells are treated in H5 media with or without TSH (10 mIU/ml). The cells are harvested at 0, 2, 4, 6, 8, 12, and 24 hours after treatment.

Thyroid papillary carcinoma cell line ONCO-DG1 (German Collection of Microorganisms and Cell Cultures [DSMZ] catalogue code ACC507) were cultured in 90% RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) and supplemented with 10% fetal calf serum (FCS), 10 µg/ml bovine insulin, 200 mM L-glutamine, 10 µM TSH, and antibiotics (Sigma Chemical Co., St. Louis, MO) in standard incubating conditions (5% CO₂, 95% humidity, 37°C). 200,000 cancer cells are seeded in 25cm² culture flask containing 5 ml regular media for 24 hours. To exclude any TSH effects, cells then are maintained for 24 hours in TSH-free H5 medium (RPMI 1640 medium supplemented with 10% FCS, 10 µg/ml insulin, 5 µg/ml human transferrine, 10µg/ml somatostatin, 2 ng/ml glycyll-histidyl-1-lysine acetate, 360µg/ml hydrocortisone, and 200mM L-glutamine) (Sigma). Thereafter, the cells are treated in H5 media with or without EGF (10ng/ml). The cells are harvested at 0, 2, 4, 6, 8, 12, and 24 hours after treatment.

**RNA extraction and cDNA synthesis**

Total RNA is extracted from tissue specimens using the TRIzol method (Gibco BRL, Gaithersburg, MD) according to the manufacture’s suggested protocol. Concentration and purity is determined using a Beckman spectrophotometer. The quality of RNA is checked by the electrophoresis of 3 µg samples in a 1.5 % agarose gel, staining with ethidium bromide. The 28S and 18S rRNA bands are examined on a UV transilluminator.

First-strand cDNA was synthesized from total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacture's instructions.250 ng RNA in volume of 50µl was combined with an equal volume of the 40U RNase inhibitor (Invitrogen, Carlsbad, CA) and 2X RT Master-Mix (Applied Biosystems, Foster City, CA), containing random primers, dNTP mixture, and Multiscribe RT enzyme. Complementary DNA is synthesized using a thermal cycler by incubating at 25 °C for 10 minutes, 37°C for 120minutes.

**Real-time quantitative PCR**

Oligonucleotide primers and Taqman probes for VEGF-C and VEGF-D are designed to be intron spanning using Primer Express software (Applied Biosystems, Foster City, CA). The following oligonucleotides were used for real-time PCR: (a) VEGF-C forward primer, 5’-TTCA
TTCCATTAGACGTTCCTCCT-3'; VEGF-C reverse primer, 5'-GATTATCCACATGTAA
TTGGTGGG-3'; VEGF-C probe, 5'-FAM CCAGCAACACTACCACAGTGCAGGCA TAMRA-3'; (b) VEGF-D forward primer, 5'-GTATGGACTCTCGTCAGC AT-3'; VEGF-D reverse primer, 5'-AGGCTCTTCATTGCAACAG -3'; (c) GUS forward primer, 5'-CTCATTTGGAATTTTGCCGATT-3'; GUS reverse primer, 5'-CCGAGTGAAGATCCCTTTTTA-3'; GUS probe, 5'-FAM TGAACAGTCACCACG
ACGAG AGTGCTGG TAMRA-3'. VEGF-D mRNA levels were measured using primer and probe sets (Hs00189521_m1) from Assays on Demand products (Applied Biosystems). Human beta-glucuronidase (GUS) gene is used as an endogenous control to normalize the expression of VEGF-C and VEGF-D. Optimal PCR conditions and PCR efficiency are determined empirically to be > 90% efficient.

Quantitative real time PCR is then performed using a 96-well optic tray on the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). A total reaction volume of 50 µl contains Universal PCR Master-Mix (Applied Biosystems, Foster City, CA), 500 nM of each primer, 200 nM probe and 10 µl cDNA (described above, equivalent to a cDNA amount from 25 ng of initial total RNA) for analyzing VEGF-C expression. 10µl cDNA was added to the Master Mix containing Assay-on-Demand Gene Expression Assay Mix (Applied Biosystems) for a final volume of 25 µl for analyzing VEGF-D expression. The PCR thermal cycle condition is setup at 50°C for 2 min, then 95 °C for 12 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The mRNA content of each target gene is simultaneously determined in the 96-well. Each sample is run as a triplicate. The negative controls lacking template RNA are included in each experiment. Data collection and analysis is performed with SDS v1.7 software (Applied Biosystems). Data is then exported and further analyzed in Excel (Microsoft, Redmond WA).

The comparative Ct method
The comparative Ct method is used. Briefly, the difference in cycle threshold (Ct, the number of PCR cycles required for the FAM (i.e., 6-carboxyfluorescein) emission intensities to exceed a threshold) between the VEGF-C, VEGF-D and GUS genes is calculated and designated as ∆Ct. VEGF-C mRNA expression, relative to GUS, is calculated according to the following formula: 2^-∆Ct. These calculations are valid since the measured PCR efficiencies are very close to 100%.

Results

Relative expression of VEGF-C/VEGF-D in TPC-1 between groups with and without TSH treatment (Fig 1-6)
From our studies, there is no significant difference of VEGF-C/VEGF-D expression in TPC-1 between groups with and without TSH treatment during 24 hr period.
Fig 1

VEGF-C expression in TPC-1

Fig 2

VEGF-C expression in TPC-1
Serial changes of VEGF-C/VEGF-D expression of two groups in TPC-1 (with and without TSH treatment) during 24 hours (Fig 7-12)
There is no upregulation of VEGF-C/VEGF-D expression in TPC-1 with TSH treatment during 24 hr period compared with those without TSH treatment although there is a mild upregulation of VEGF-C/VEGF-D in both groups during 24 hour culture.
Fig 7

**VEGF-C expression in TPC-1**

![Graph showing VEGF-C expression in TPC-1 with relative expression on the y-axis and hours on the x-axis. The graph compares the expression at 0 mU/ml TSH and 10 mU/ml TSH.](image)

Fig 8

**VEGF-C expression in TPC-1**

![Graph showing VEGF-C expression in TPC-1 with relative expression on the y-axis and hours on the x-axis. The graph compares the expression at 0 mU/ml TSH and 10 mU/ml TSH.](image)
Fig 9

VEGF-C expression in TPC-1

Fig 10

VEGF-C expression in TPC-1
**Relative expression of VEGF-C/VEGF-D in ONCO-DG-1 with and without EGF treatment (Fig 13-17)**

From our studies, there is no significant difference of VEGF-C/VEGF-D expression in ONCO-DG-1 between groups with and without EGF treatment during 24 hr period.
Fig 15

**VEGF-C expression in ONCO-DG1**

![VEGF-C expression graph](image)

- **0 ng/mL EGF**
- **10 ng/mL EGF**

Fig 16

**VEGF-D expression in ONCO-DG1**

![VEGF-D expression graph](image)

- **0 ng/mL EGF**
- **10 ng/mL EGF**
Serial changes of VEGF-C/VEGF-D expression of two groups in ONCO-DG-1 (with and without EGF treatment) during 24 hours (Fig 18-22)

There is no upregulation of VEGF-C/VEGF-D expression in ONCO-DG-1 with EGF treatment during 24 hr period compared with those without EGF treatment.
**Fig 19**

VEGF-C expression in ONCO-DG1

- 0 ng/mL EGF
- 10 ng/mL EGF

**Fig 20**

VEGF-C expression in ONCO-DG1

- 0 ng/mL EGF
- 10 ng/mL EGF
Discussion
Thyroid stimulating hormone (TSH) is documented to stimulate tumor growth and metastasis in thyroid cancers. To suppress TSH is thought to be able to control thyroid tumor growth and metastasis. Clinically, thyroid suppression therapies using thyroid hormone have been applied to the treatment of benign thyroid nodules and control of the malignant diseases postoperatively. Soh et al. have reported TSH may promote growth in some thyroid cancers by stimulating VEGF secretion and angiogenesis. In vivo study from Viglietto et al. also demonstrated that in the thyroid gland of thiouracil-fed rats, increased mRNA and protein expression of VEGF and Flk-1/KDR occurred subsequent to the rise in the serum TSH levels and in parallel with thyroid capillary proliferation.
In this investigation, we try to determine whether TSH may alter the VEGF-C/VEGF-D expression in papillary thyroid cancers, thus cause its propensity for lymph node metastasis. In this study, nevertheless, we can not find the evidence of upregulation of VEGF-C/VEGF-D expression in TPC-1 with TSH treatment. The effect of TSH on lymphangiogenesis through VEGF-C/VEGF-D in thyroid cancers could not be established.

Thyroid cancers are known to arise and proliferate in an epidermal growth factor (EGF)-rich environment and possess EGF receptors. Overexpression of epidermal growth factor receptor and related erb-B receptor tyrosine kinase has been demonstrated in many human tumors. The presence of erb-B family receptors has been demonstrated in normal and neoplastic thyroid tissue, as well as thyroid cancer cell line. Stimulation of thyroid cancer cell lines with epidermal growth factor was shown to enhance in vitro metastasis. However, the correlation of epidermal growth factor and lymphangiogenic factors, VEGF-C/VEGF-D, in thyroid cancer has not been clearly clarified.

In this investigation, we also try to determine whether epidermal growth factor may alter the VEGF-C/VEGF-D expression in papillary thyroid cancers, thus cause its propensity for lymph node metastasis. Nevertheless, we can not find the evidence of upregulation of VEGF-C/VEGF-D expression in ONCO-DG-1 with EGF treatment. The effect of EGF on lymphangiogenesis through VEGF-C/VEGF-D in thyroid cancers could not be established, too.

**Conclusion**

Thyroid stimulating hormone and epidermal growth factor, which stimulates the expression of many genes in thyroid cancer cells, does not stimulate VEGF-C/VEGF-D mRNA expression in thyroid papillary cancer cell line, TPC-1 and ONCO-DG-1.

The stimulating and inhibiting factors of VEGF-C/VEGF-D in thyroid papillary cancers need to be further studied.