行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：　個別型計畫　　□整合型計畫

計畫編號：NSC 90－2314－B－002－253－

執行期間：　90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：張天鈞

共同主持人：王治元

共同主持人：李文森

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執行單位：國立台灣大學醫學院內科

中　華　民　國　　91　年　9　月　16　日

行政院國家科學委員會專題研究計畫成果報告
細胞激素對甲狀腺未分化癌再分化及細胞凋亡的影響
The effects of cytokines on the differentiation and apoptosis of anaplastic thyroid cancer

計畫編號：NSC 90－2314－B－002－253－
執行期限：90 年 8 月 1 日至 91 年 7 月 31 日
主持人：　張天鈞　國立台灣大學醫學院內科
共同主持人：王治元　國立台灣大學醫學院生理研究所
共同主持人：李文森　私立台北醫學院生理科

中文摘要

甲狀腺未分化癌的病人幾乎在很短的時間就會死亡，因此有必要研究出治療的方法。讓細胞由未分化轉變成分化是可行的辦法之一。由於在分化時細胞膜會出現微細絨毛的現象，本研究乃在觀察腫瘤壞死因子α對未分化甲狀腺癌細胞型態之影響，並探討其機轉。我們將未分化甲狀腺癌細胞株用腫瘤壞死因子α處理，然後再用電子顯微鏡觀察其細胞之變化。為瞭解其作用機轉，我們用免疫墨點法來分析抑制性κB 蛋白，並用電泳移動轉移試驗來分析核因子之活化。我們也探討核因子κB (NF-κB)轉位抑制劑 - NF-κB SN50 對腫瘤壞死因子α誘導甲狀腺未分化癌細胞型態學變化之影響。此外我們也測定培養液中之甲狀腺球蛋白及血管內皮細胞生長因子的值。結果顯示腫瘤壞死因子α可以誘導NF-κB 之活化，而此活化和轉位入細胞核是讓未分化甲狀腺癌細胞產生分化的原因，這種現象可以被 NF-κB 轉位抑制劑 NF-κB SN50 所抑制。腫瘤壞死因子α也可以促進未分化癌細胞分泌甲狀腺球蛋白和減少血管內皮細胞生長因子的分泌。總之，腫瘤壞死因子α可以經由 NF-κB 誘導未分化甲狀腺癌細胞的分化，值得進一步探討是否可以用來治療未分化癌，甲狀腺細胞微細絨毛是研究未分化甲狀腺癌分化的有用指標。

關鍵詞：腫瘤壞死因子α、未分化甲狀腺癌、細胞型態、核因子κB、分化

Abstract

Anaplastic thyroid cancer is almost uniformly fatal. Microvilli are an important three-dimensional (3-D) cytomorphological feature of thyrocyte differentiation, as fewer microvilli being seen in less differentiated cancer. Differentiation therapies, such as retinoic acid and somatostatin, have been testd in differentiated thyroid cancer experimental models, but not on anaplastic thyroid cancer before. The aim of this study was to elucidate whether TNF-α can induce 3-D cytomorphological differentiation of anaplastic thyroid cancer cells, and, if so, to investigate the mechanism involved. Anaplastic thyroid cancer cells were treated with TNF-α and examined for evidence of cytomorphological differentiation using electron microscopy. To study the mechanism of differentiation, immunoblotting was used to analyze I-κB proteins and electrophoretic mobility shift assays to analyze NF-κB activation. The effect of NF-κBSN50, a NF-κB translocation inhibitor, on cytomorphological changes induced in anaplastic thyroid cancer cells by TNF-α was also studied. Meanwhile, levels of thyroglobulin and vascular endothelial growth factor (VEGF) secreted into the culture medium were also measured. Our results showed that TNF-α can induce activation of NF-κB and that the activation and translocation of NF-κB

into the nucleus is responsible for promoting the 3-D cytomorphological differentiation of anaplastic thyroid cancer cells, which could be inhibited by the NF-κB translocation inhibitor, NF-κB SN50. TNF-α also induced increased thyroglobulin secretion and reduced VEGF secretion by anaplastic cancer cells. Our data suggest that TNF-α can induce thyrocyte differentiation in anaplastic thyroid cancer cells through NF-κB and that it merits investigation as differentiation therapy for the treatment of anaplastic thyroid cancer. We also found that microvilli to be a useful marker for studying thyrocyte differentiation in thyroid anaplastic cancer cells.

Keywords: tumor necrosis factor α, anaplastic thyroid carcinoma, cytomorphology, nuclear factor κB, differentiation

Introduction

Cellular dedifferentiation is very common in malignant transformation, occuring in up to one-third of differentiated thyroid cancers [1]. Normal thyroid epithelial cells are usually uniform in size and shape, and microvilli can be easily observed in the three-dimensional (3-D) cytomorphology by scanning electron microscopy (SEM) [2-5].. Microvilli are fingerlike extensions found on the surface of many animal cells, being particularly abundant on epithelial cells, which require a very large surface area to function efficiently. Studying cancerous specimens, Nesland et al [2] found the major difference in thyroid carcinomas to be variations in microvilli abundance. In neoplastic states, the abundance of microvilli steadily decreases on going from ordinary papillary thyroid carcinoma through follicular variants of papillary thyroid carcinoma to follicular carcinoma, and anaplastic carcinoma thyrocytes have few or no microvilli [2]. Microvilli are therefore a 3-D cytomorphological feature of thyrocyte differentiation, as fewer microvilli being seen in less differentiated cancers [6].

TNF-α, produced primarily by macrophages, regulates the expression of genes which play pivotal roles in immunologically mediated inflammatory responses [7,8]. The ubiquitous nuclear factor [kappa] B (NF-κB) family includes at least five members, NF-κB1. NF-κB2, RelA, RelB, and c-Rel [9]. Activation of NF-κB by TNF-α is very important because many TNF-α-regulated genes contain binding sites for NF-κB. Normally, NF-κB is sequestered in the cytoplasm by inhibitory proteins, the I-κBs (I-κBα, I-κBβ, I-κBγ, and I-κBε), but cellular stimulation leads to phosphorylation of the I-κBs by specific I-κB kinases and the dissociation of I-κB from the NF-κB/I-κB complex [10,11].. NF-κB not only induces the expression of many genes related to the processes of immunity and inflammation [12], but also regulates apoptosis [13,14].. Studies on B cells from NF-κB knockout mice, dendritic cells, macrophages, small cell lung cancer cells, and neuroblastoma cells have indicated that it also plays a critical role in cellular differentiation and development [15-19]..

Although only 1% of differentiated thyroid cancers transform into anaplastic thyroid cancer, this disease is almost always fatal [20]. Several studies on redifferentiation agents, including retinoic acid and somatostatin [21,22], have been performed using a differentiated thyroid cancer experimental model. However, no reports on differentiation in the field of anaplastic thyroid cancer have been published. Here, we show that TNF-α can induce the appearance of microvilli in an anaplastic thyroid cancer cell line (ARO), and NF-κB, activated by TNF-α, is required to promote the differentiation of thyroid follicular cells, and that this process can be blocked by NF-κB SN50 [23], a cell-permeant peptide which inhibits the translocation of NF-κB into the nucleus of stimulated cells by specifically binding to the nuclear localization signal for NF-κB.

Vascular endothelial growth factor (VEGF) is an angiogenic factor that plays

important roles in tumor growth. Previous studies have shown that VEGF expression correlates with tumor growth and metastasis, including thyroid cancer [24-28]. In addition, the secretion of thyroglobulin, a glycoprotein produced only by normal or neoplastic thyroid follicular cells is a specifically differentiated function in thyrocytes [29,30].. We therefore measured levels of thyroglobulin and VEGF in culture medium from untreated and TNF-α-treated ARO cells as further parameters of thyrocytes differentiation.

Materials and Methods

Cell Culture and Reagents

Human ARO anaplastic thyroid cancer cells, kindly provided by Dr. S.D. Chen (Chang-Gung Memorial Hospital, Taiwan), were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin, and 0.1 μg/ml of streptomycin. For differentiation, 1x 10^5 cell/ml were incubated in 10 cm cell culture plates for 24 hours, then 5 ng/ml of human recombinant TNF-α (Boehringer Mannheim) was added, and the 3-D cytomorphology observed after 24 and 48 hours. To test the effect of inhibition of NF-κB translocation into the nuclei of stimulated cells, 50 µg/ml of NF-κB SN50 (BIOMOL, Hamburg, Germany) was added to the cells during TNF-α treatment.

Preparation of Cytoplasmic and Nuclear Proteins

Cells were harvested and washed in ice-cold phosphate-buffered saline. To prepare cytoplasmic proteins, the cell pellets were suspended in ice-cold lysis buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml each of aprotinin and leupeptin), incubated for 30 min on ice, centrifuged for 5 min at 200 g at 4ºC, and the supernatants collected. To isolate nuclear proteins, cells were homogenized at 4ºC in 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM KCl, 1 mM EDTA, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml of protease inhibitors, incubated at 4ºC for 20 min with occasional vortexing, centrifuged at 16,000 g for 20 min at 4ºC, and the supernatants collected.

Electrophoretic Mobility Shift Assays (EMSAs)

Nuclear proteins and the ^{32}P-labeled double-stranded oligonucleotide probe, 5'-AGTTGAGGGGACTTTCCCAGGC-3', were prepared (the underlining indicates the κB consensus sequence or binding site for NF-κB/c-Rel homodimeric and heterodimeric complexes). For EMSAs, 2 μg of nuclear extract was mixed with the labeled probe and incubated at room temperature for 20 min, then the DNA/protein complex was separated on 6% non-denaturing acrylamide gels before vacuum drying and autoradiography. Specificity of binding was tested by competition with the unlabeled oligonucleotide.

Western Blot Analysis

30 μg samples of cytoplasmic proteins were electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was carried out with antibodies specific for I-κBα (100-4176C, Rockland) in phosphate-buffered saline with 0.2% Tween 20 (Sigma) and 5% bovine serum albumin (Sigma). Specific proteins were visualized using the enhanced chemiliminescence method (Amersham Pharmacia Biotech).

SEM and Transmission Electron Microscopy (TEM)

For SEM, ARO cells on albumin-coated coverglasses were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h, then the slide was washed for 3 x 5 min with cacodylate buffer, pH 7.4, fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 30 min, and washed for 3 x 5 min with 0.1 M cacodylate buffer, pH 7.4. The specimen was dehydrated successively for 5 min with 70%, 85%, and 95% alcohol, then for 3 x 5 min with 100% alcohol. Critical point drying was performed

using a HCP2 critical point drier (Hitachi, Tokyo, Japan), then the specimen was coated with Au-Pd with a JEC-1100 ion spatter (Japan Electron Optical Laboratory, Tokyo, Japan) and observed with a JSM-T330A scanning microscope (Japan Electron Optical Laboratory).

For TEM, ARO cells treated with TNF-α were fixed for 1 hour in 4% glutaraldehyde, washed for 3 x 10 min with 0.1 M cacodylate buffer, pH 7.4, fixed for 30 min with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, and again washed for 3 x 10 min with 0.1 M cacodylate buffer, pH 7.4. The specimen was dehydrated successively with 70%, 85%, and 95% alcohol (10 min each), then for 3 x 10 min with 100% alcohol. The dehydrated specimen was immersed in propylene oxide for 2 x 10 min, then in a 1:1 propylene oxide:Epon mixture for 1 hour, a 1:3 propylene oxide:Epon mixture for 2 hours, and pure Epon overnight, before being embedded in a commercially available capsule and polymerized in a 60°C oven for 48 hours. Ultrathin sections (1 μm thick) were cut using an Ultracut E ultramicrotome (Reichert - Jung, Vienna, Austria) and mounted on copper grids. The sections were subjected to electron staining with uranium acetate and lead citrate for 5 min each, then observed using a JEM-2000EXII electron microscope (Japan Electron Optical Laboratory).

Thyroglobulin by Radioimmunoassay (RIA)

Triplicate samples of culture medium were collected from TNF-α treated and untreated ARO cells (10^5) at 24, 48, and 72 hours and assayed for thyroglobulin using a RIA kit (Double Antibody Thyroglobulin, EURO/DPC Ltd. Glyn Rhonwy, Gwynedd, United Kingdom) for the quantitative measurement of thyroglobulin.The sample was first preincubated with the anti-thyroglobulin antiserum, then ^{125}I-labled thyroglobulin was added. After incubation for a fixed time, bound and free label were separated by the double-antibody method, and the antibody-bound fraction precipitated and count. Sample concentrations are read from a calibration curve. The tracer has a high specific activity, with total counts of 35,000 cpm at iodination. Maximum binding is approximately 40% and nonspecific binding negligible.

VEGF by Enzyme-Llinked Immunosorbent Assay (ELISA)

Triplicate samples of culture medium were collected from TNF-α treated and untreated ARO cells (10^5) at 24, 48,and 72 hours and assayed for VEGF by ELISA, using a kit (Human VEGF Colorimetric ELISA, Pierce Endogen, Rockford, IL, U.S.A.) designed to measure natural and recombinant $VEGF_{165}$. The optical density was measured using an ELISA reader at 450 nm. The assay range was 31.3 to 2,000 pg/ml and the intra- and inter-assay variance was 5.5 % and 9.1 %, respectively.

Statistical Analysis

VEGF and thyroglobulin levels in untreated and treated ARO cell culture supernatant were compared by the paired student's *t*-test. A *P* value of < 0.05 was considered statistically significant.

Results

TNF-α treatment results in enhanced I-κBα degradation

Normally, NF-κB is sequestered in the cytoplasm by inhibitory proteins, the I-κB. Various stimuli induce degradation of I-κB proteins, resulting in the release and nuclear translocation of NF-κB [9]. Since a previous report indicated that TNF-α preferentially targets I-κBα [24], we first investigated the effect of TNF-α on I-κBα degradation. Western blot analysis of the levels of I-κBα proteins in cytoplasmic extracts showed that, in ARO cells, 20 min of treatment with 5 ng/ml of TNF-α resulted in a reduction in I-κBα levels.

TNF-α activates NF-κB nuclear translocation

To determine the effect of TNF-α -induced NF-κB DNA binding activity *in vitro*, ARO cells were treated with 5 ng/ml of TNF-α and nuclear extracts analyzed by EMSA.. As expected, there was very little binding to the consensus κB site using a nuclear extract from untreated ARO cells, but TNF-α treatment for 30, 60, or 100 min resulted in detection of the major NF-κB complex.

3-D cytomorphological differentiation of ARO cells

Untreated ARO cells usually showed a disorganized cellular arrangement with a smooth cellular surface and an absence of microvilli on SEM, whereas, after treatment with 5 ng/ml of TNF-α for 24 or 48 hours, a more uniform cell population with distinct borders and an abundance of microvilli was seen.. After 24 hours treatment with 5 ng/ml of TNF-α, microvilli and cytoplasmic dense-core secretory granules were also identified by TEM.

NF-κB SN50 inhibits NF-κB translocation and ARO cell differentiation

Cotreatment with TNF-α and NF-κB SN50, a cell-permeant peptide which inhibits NF-κB translocation into the nucleus, was carried out. As expected, after 30 min treatment with 5 ng/ml of TNF-α and 50 μg/ml of NF-κB SN50, the major NF-κB complex was not detected.. In addition, SEM and TEM showed a disorganized cellular arrangement with a smooth cellular surface and an absence of microvilli, as seen in untreated ARO cells. This result suggests that the activation and translocation of NF-κB into the nucleus is responsible for promoting the differentiation of anaplastic cancer cells.

Measurement of thyroglobulin secretion by RIA

All cancer cells secreted thyroglobulin into the conditioned medium as determined by RIA. TNF-α-treated ARO cells secreted much more thyroglobulin protein than untreated cells .

Measurement of VEGF secretion by ELISA

All cancer cells secreted VEGF into the conditioned medium as determined by ELISA； however, the amount secreted by TNF-α-treated ARO cells was significantly lower.

Discussion

Our present study showed that treatment of human anaplastic thyroid cancer cells with the cytokine, TNF-α, caused a reduction in the levels of the NF-κB inhibitor, I-κB, and resulted in NF- κB translocation and cell differentiation, both of these last two effects being blocked by NF-κB SN50, a cell-permeant peptide which inhibits NF-κB translocation into the nucleus. Although 72 hours' treatment with TNF-α did not result in marked changes in growth rate and apoptotic rate, immunoblotting showed increased expression of the adhesion receptor, intracellular adhesion molecule (ICAM), after only four hours of TNF-α treatment (data not shown). Since downregulation of adhesion molecules usually associates with tumor dedifferentiation and infiltrative growth, this upregulation is consistent with differentiation.

TNF-α is known to be involved in many biological responses and the above results provides evidence that it is also involved in the differentiation of thyroid anaplastic cancer cells. In a previous report, the major difference in the differentiation of thyroid cancers was the variation in the abundance of microvilli [2]. Microvillous patterns are important 3-D cytomorphological features of thyrocyte differentiation, as fewer microvilli are seen in less differentiated cancers [6]. The present study showed that TNF-α can trigger microvilli formation in human anaplastic thyroid cancer cell lines.

The transcription factor, NF-κB, plays an important role during an immune response, since it controls the transcription of various genes required to mount an effective response. Several studies have indicated that it is also involved in cellular proliferation and

differentiation [31-33].. In the present study, we found that thyroid anaplastic cancer cell differentiation was closely related to NF-κB activation, since differentiation was blocked by an NF-κB translocation. However, a previous report[33] indicated that increased levels of NF-κB are associated with oncogenesis and that the ability of NF-κB to protect cells against chemotherapeutic drugs or TNF-induced apoptosis suggested that NF-κB plays an important role in cell survival. In addition, activation of NF-κB proteins has also been observed during neuronal differentiation in brain and neuroblastoma cells [18].. This observation is consistent with the characteristics of NF-κB expression in multiple immunological pathways, i.e. functional overlap may mask other important roles of NF-κB.

The 3-D cytomorphological differentiation features of thyroid anaplastic cancer cells after TNF-α treatment reported in this study may be of use in management of anaplastic thyroid cancer. Since thyroid anaplastic cancer is refractory to conventional chemotherapy and is usually fatal, these cytomorphological features could provide early information on differentiation, allowing I-[131] radiotherapy to be used. On the other hand, chemotherapy combined with surgical ablation and radioiodine ablation may be more effective after differentiation therapy, and may increase the survival rate of such patients.

In differentiated thyroid cancers, iodine uptake and metabolism, TSH receptor expression, thyroglobulin synthesis and secretion, and thyroid peroxidase activity have been reported as markers of thyrocyte differentiation [34-36]. In addition, VEGF is an important angiogenic factor, and increasing expression of VEGF correlated with tumor growth and metastasis[24-28].. In the present study, we also measured thyroglobulin and VEGF levels in the culture medium and found that TNF-α-treated ARO cells secreted more thyroglobulin and less VEGF tha untreated cell, thus providing further support for the cytomorphological differentiation of anaplastic thyroid cancer cells.

In conclusion, our data show that TNF-α can induce thyrocyte differentiation in anaplastic thyroid cancer cells, as shown by 3-D cytomorphological changes in microvilli, increased thyroglobulin secretion, and decreased VEGF secretion. Activation and translocation of NF-κB into the nucleus is responsible for promoting the differentiation of anaplastic cancer cells. The result suggest that TNF-α merits further investigation as differentiation therapy for treatment of anaplastic thyroid cancer.

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