Thymosin α1 Down-regulates the Growth of Human Non-Small Cell Lung Cancer Cells in Vitro and in Vivo

Terry W. Moody,1 Mirela Fagarasan, Farah Zia, Mirjana Csesnjaj, and Allan L. Goldstein

Department of Biochemistry and Molecular Biology, The George Washington University School of Medicine and Health Sciences, Washington, DC 20037 [T. W. M., M. F., F. Z., A. L. G.], and Laboratory of Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892 [M. C.]

ABSTRACT

The effect of thymosin α1 (THNα1) and its NH2-terminal fragment (THN1–14), and COOH-terminal fragment (THN15–28), on non-small cell lung cancer (NSCLC) growth was evaluated. Using an anti-THNα1 antibody, receptors were identified on NSCLC cells that were pretreated with 10–4 M THNα1. [3H]Arachidonic acid was readily taken up by NSCLC cells and THNα1 significantly increased the rate of arachidonic acid release. THN1–14 slightly stimulated but THN15–28 and THNB4 did not alter arachidonic acid release from NCI-H1299 cells. In clonogenic growth assays in vitro, THNα1 (10–4 M) significantly decreased NSCLC colony number whereas THN1–14, THN15–28, and THNB4 were less potent. Using growth assays in vivo, THNα1 (10 µg s.c./day) but not THN1–14, THN15–28, or THNB4 inhibited significantly NSCLC xenograft formation in nude mice. These data suggest that biologically active THNα1 receptors are present on NSCLC cells and that native THNα1 inhibits the growth of human NSCLC.

INTRODUCTION

THNα1,1 a 28-amino acid peptide which functions as a biological response modifier by virtue of its immunomodulatory effects on T-cells, was initially isolated from a crude bovine thymus extract, THN fraction 5 (1). THNα1 is derived from a 113-amino acid precursor protein, prothymosin α; the NH2-terminal 28-amino acid sequence of prothymosin α is identical to that of THNα1 (2, 3). By radioimmunoassay THN-like peptides have been detected in numerous tissues including the rat thymus, pituitary, and brain (4). In addition to THNα1, THNβ4, a 43-amino acid peptide with pleotropic effects as a biological response modifier, was isolated from THN fraction 5 (5). THNβ4, which has little sequence homology to THNα1, is an actin-sequestering peptide, stimulates terminal deoxynucleotidyltransferase, and stimulates luteinizing hormone-releasing factor secretion from the rat hypothalamus resulting in increased luteinizing hormone secretion from the pituitary (5–7).

THNα1 has been localized to the surface of mouse thymic epithelial cells using anti-THN antibodies (8). While the second messenger for THNα1 is not known, THN fraction 5 stimulated arachidonic acid release from anterior pituitary cells (9). Also, THNα1 has sequence homology to VIP (10). THNα1 is reported to inhibit 125I-VIP binding sites in rat blood mononuclear cells and liver plasma membranes with low affinity (50% inhibitory concentration, 1 µM). These studies suggest that THNα1 can interact with VIP receptors. Previously we found 125I-VIP binding sites on human lung cancer cells (11, 12).

THN fraction 5 prolongs survival of some lung cancer patients (13). In postradiotherapy patients with NSCLC, synthetic THNα1 accelerated reconstitution of thymic-dependent immunity (14). Also, THNα1 increased survival especially in patients with nonbulky tumors (15). THNα1 in combination with interferon after cyclophosphamide treatment increased survival of mice with Lewis lung carcinomas (16). These data suggest that THNα1 inhibits lung cancer proliferation. Here the effects of THN-like peptides on NSCLC cells were investigated.

MATERIALS AND METHODS

Cell Culture. NSCLC cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum. When a monolayer formed, the adherent cells were washed with PBS and treated with trypsin/EDTA. The cells were pelleted and resuspended in serum supplemented medium and incubated at 37°C in 5% CO2/95% air (17). Routinely the cells were passaged 1/1 weekly and experiments were conducted when the cells were in exponential growth phase.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 5/24/93; accepted 8/27/93.

1 To whom requests for reprints should be addressed, at Biomarkers and Prevention Res. Bz., NCI, 9610 Medical Center Dr., Bldg. C, Rm. 300, Rockville, MD 20850.

2 The abbreviations used are: THNα1, thymosin α1; VIP, vasoactive intestinal peptide; PBS, phosphate-buffered saline; NSCLC, non-small cell lung cancer; BSA, bovine serum albumin; EGF, epidermal growth factor; cAMP, cyclic AMP; TGFα, transforming growth factor α.

5214
right flank of each mouse by s.c. injection. Palpable tumors were observed in approximately 90% of the mice after 2 weeks. PBS (100 μl) or THN-like peptides (10 μg/day) were injected s.c. adjacent to the tumor. The tumor volume (height x width x depth) was determined weekly by calipers and recorded. When the tumor became necrotic, the growth studies were terminated.

RESULTS

Immunocytochemistry. Previously, we found that THNα1 antiserum recognized the COOH terminus of THNα1 whereas the NH2 terminus was essential for biological activity (4). Here NSCLC cells were fixed with glutaraldehyde and treated with a primary antibody (NSP or PR-1), followed by a secondary antibody (goat anti-rabbit serum) and silver enhancer. Using large cell carcinoma cells (NCI-H1299) optimal staining was observed using a 1:1000 dilution of NSP or PR-1. Cells did not stain in the absence of THNα1 antiserum (Fig. 1A) or THNα1 treatment (Fig. 1B). NCI-H1299 cells immunostained if they were treated with 1 μm THNα1, followed by extensive washing, fixation, and treatment with primary and secondary antibody (Fig. 1C). These data suggest that THNα1 binds to NSCLC cell surface component. Similar data were obtained using adenocarcinoma (NCI-H838), squamous cell carcinoma (NCI-H157), and lung carcinoid cells (NCI-H727).

Arachidonic Acid. Previously it was found that THN fraction 5 stimulated arachidonic acid release from pituitary cells (9). Here the effects of THN-like peptides on arachidonic acid release were investigated using NSCLC cells. THNα1 had little effect on arachidonic acid release at a 10^{-9} M concentration using NCI-H1299 cells but significantly stimulated arachidonic acid release at 10^{-6} M concentration (Fig. 2). THNα1 (10^{-5} M) increased the rate of arachidonic acid release approximately 3-fold and the half-maximal effective concentration was 0.7 × 10^{-7} M. Table 1 shows that THN1-14 (10^{-6} M) slightly increased arachidonic acid release, respectively, whereas THN1-28 and THN84 had no effect. Similar results were obtained using NCI-H727, 838, and 1264 cells.

NSCLC Proliferation. The effect of THNα1 on NSCLC growth was investigated in vitro. Fig. 3 (top) shows that 150 NCI-H1299 colonies formed in the control culture. When 100 nM VIP was added the number of colonies formed increased significantly to 322 (data not shown). THNα1 inhibited NCI-H1299 colony formation in a dose-dependent manner with 10^{-7}, 10^{-6}, or 10^{-5} but not 10^{-8} M THNα1 causing significant inhibition. Fig. 3 (middle) shows that numerous large viable colonies formed. In the presence of 1 μM THNα1 [Fig. 3 (bottom)] the number of colonies was reduced as was the size. Similar data were obtained using NSCLC cell lines NCI-H157, H727, H838, and H1264.

Table 2 shows that other THN-like peptides inhibited NCI-H838 colony formation. THN1-14 and THN15-28 inhibited colony formation by approximately 25% whereas THN84 was inactive. These data suggest that NH2- and COOH-terminal fragments of THNα1 have some biological activity but neither fragment is as potent as is native THNα1.

Also, the effects of THNα1 on NSCLC growth was investigated in vivo. Fig. 4 shows that tumors formed 1 week after s.c. injection of NCI-H157 cells into nude mice. Fig. 4 (top) shows that the NSCLC xenografts grew exponentially and after 5 weeks the xenograft volume was 2306 mm^3. When THN1-14 or THN15-28 was administered s.c. (10 μg/day) xenograft volume was reduced by approximately 20%. In contrast, THNα1 (10 μg) significantly reduced tumor volume by approximately 75%. A dose-response curve for THNα1 was determined. Fig. 4 (bottom) shows that 10 or 1 μg of THNα1 significantly decreased NCI-H727 tumor volume whereas 0.1 μg of THNα1 or 10 μg of THN84 were ineffective. The effects of THNα1 were reversible in that if treatment was discontinued, the growth rate of the tumor returned to normal values.

DISCUSSION

Previously, THNα1 was postulated to prolong survival of NSCLC patients via effects of the immune system (15). Here THNα1 inhibited the growth of NSCLC cells in vitro and in vivo suggesting that THNα1 directly interacts with NSCLC cells. (125I-Tyr9)THNα1 binds with high affinity to THNα1 antiserum but not NSCLC cells. Additional studies indicated that THNα1 and THN15-28 but not THN1-14 strongly cross-reacted with the antiserum.
Fig. 2. Effect of THNα-1 on arachidonic acid release. NCI-H1299 cells were loaded with [3H]arachidonic acid and the amount of radiolabeled arachidonic acid released was determined with increasing concentrations of THNα-1 (▲). This experiment is representative of 2 others. Bars, SD.

Table 1 Effect of THN-like peptides on arachidonic acid release

<table>
<thead>
<tr>
<th>Agent added</th>
<th>[3H]Arachidonic acid released (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>688 ± 90*</td>
</tr>
<tr>
<td>THNα1, 10^{-6}M</td>
<td>1734 ± 72b</td>
</tr>
<tr>
<td>THN^{1-14}, 10^{-6}M</td>
<td>855 ± 63</td>
</tr>
<tr>
<td>THN^{15-28}, 10^{-6}M</td>
<td>602 ± 51</td>
</tr>
<tr>
<td>THN^{84}, 10^{-6}M</td>
<td>608 ± 168</td>
</tr>
</tbody>
</table>

* Mean ± SE of 4 determinations using NCI-H1299 cells.

b \( P < 0.01 \).

c \( P < 0.001 \).

Table 2 Effect of THN-like peptides on NSCLC growth in vitro

<table>
<thead>
<tr>
<th>Agent added</th>
<th>Colony no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>54 ± 4*</td>
</tr>
<tr>
<td>THN^{15-28} (1 μM)</td>
<td>44 ± 4</td>
</tr>
<tr>
<td>THN^{1-14} (1 μM)</td>
<td>40 ± 3#</td>
</tr>
<tr>
<td>THN^{84} (1 μM)</td>
<td>52 ± 5</td>
</tr>
<tr>
<td>THNα1 (1 μM)</td>
<td>17 ± 4</td>
</tr>
</tbody>
</table>

* The mean ± SE of NCI-H838 colonies (n = 3) was determined.

b \( P < 0.05 \).

c \( P < 0.01 \).

Therefore a unique immunocytochemical approach was used to demonstrate interaction of THNα1 with NSCLC cells. Using this assay, THNα1 was bound to NSCLC cells and fixed with glutaraldehyde (the NH₂ terminus of THNα1 may bind to a receptor protein whereas the COOH terminus was in solution and consequently interacted with THNα1 antibodies). Then THNα1 antisera was added, followed by goat anti-rabbit sera, and the bound antibodies were visualized by silver staining. These data suggest that THNα1 may interact with a NSCLC cell surface component, possibly a THNα1 receptor. Also, THNα1 can bind to the VIP receptor inasmuch as THNα1 inhibits 125I-VIP binding to NSCLC cells with low affinity (50% inhibitory concentration, 10 μM). \(^3\) Previously we found that almost all NSCLC cell lines examined have high affinity binding sites for EGF (19) and VIP (20); the VIP and EGF receptor proteins are composed of 459- and 1186-amino acid residues, respectively (21, 22).

The ability of THNα1 to alter second messenger production was determined. THNα1 (10 μM) did not alter intracellular cAMP, whereas 10 nM VIP strongly increased cAMP levels (20). THNα1 did not alter tyrosine phosphorylation whereas EGF (100 ng/ml) activated tyrosine kinase activity causing phosphorylation of the Mr 170,000 EGF receptor (23). Also, THNα1 had no effect on phosphatidylinositol turnover or cytosolic calcium but did significantly stimulate arachidonic acid release. The effect was dose dependent and THNα1 significantly stimulated [3H]arachidonic acid release. THN^{1-14} slightly stimulated arachidonic acid release whereas THN^{15-28} and THN^{84} did not alter arachidonic acid release. These data suggest that THNα1 may stimulate phospholipase A₂ but not adenylate cyclase activity or tyrosine kinase activity. Previously, it was found that NSCLC cells have cy-
and was less effective if injected i.p. or i.v. It is possible that THNα1 is degraded by serum proteases and we are currently investigating the half-life of THN-like peptides in the blood. Recently, we found that THNamide, which has an amidated COOH terminus and is resistant to degradation by carboxypeptidases, strongly inhibits NSCLC growth. VIP, which has an amidated COOH terminus (28), is also resistant to degradation by carboxypeptidases.

It remains to be determined if THN-like peptides function as autocrine growth factors in NSCLC. By radioimmunoassay, THN-like peptides are present in NSCLC extracts and conditioned medium. Previously, TGFα mRNA was found in NSCLC cells and TGFα immunoreactivity was found in conditioned medium (29). TGFα binds with high affinity to EGF receptors, causes tyrosine kinase activity, and stimulates growth (23). Because EGF receptor monoclonal antibodies inhibit the growth of NSCLC, TGFα may be a positive autocrine growth factor (19). Currently, we are investigating if THNα1 is a negative autocrine growth factor for NSCLC.

THNα1 may also be effective at slowing the growth of other cancers. In small cell lung cancer, VIP elevates cAMP levels, stimulates early oncogene expression of c-fos, and increases the secretion rate of bombesin-like peptides resulting in increased small cell lung cancer growth (30). THNα1 (10 μM) inhibited the clonal growth of several small cell lung cancer cell lines (20).

In summary, THNα1 stimulates arachidonic acid release and inhibits NSCLC growth in vitro and in vivo. Therefore THNα1 may function as a regulatory peptide in NSCLC.

REFERENCES


THYMOSIN α1 DOWN-REGULATES GROWTH OF HUMAN NSCLC


Thymosin α1 Down-regulates the Growth of Human Non-Small Cell Lung Cancer Cells in Vitro and in Vivo


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/21/5214

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/53/21/5214. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.