The effect of thalidomide on non-small cell lung cancer (NSCLC) cell lines: possible involvement in the PPARγ pathway

Kathleen L.DeCicco, Takemi Tanaka, Fausto Andreola

Carcinogenesis vol.25 no.10 pp.1805-1812, 2004
doi:10.1093/carcin/bgh210

Introduction

Lung cancer mortality accounts for over 157 000 deaths per year, making it the leading cause of cancer-related death in developed countries (1). The most common form of lung cancer, non-small cell lung cancer (NSCLC), represents ~80% of the total cases and comprises four subtypes, namely adenocarcinoma, adenosquamous carcinoma, squamous cell carcinoma and large cell carcinoma (LCC) (2). Of these subtypes, LCC tumors, classified by a complete lack of any differentiation markers, generally grow and metastasize at an earlier stage than other forms of NSCLC (2). Considering the high metastatic rate of this type of lung cancer, prognosis is poor, with a 10–15% 5-year survival rate. Since tumor growth and metastasis are angiogenesis-dependent, attempts have been made to target tumor vasculature through the use of anti-angiogenic drugs, some of which are now in phase I–III clinical trials (3,4).

Thalidomide is an anti-angiogenic (5) and immunomodulatory (6) drug that was first marketed in Europe in the late 1950s for the treatment of pregnancy-associated morning sickness. It subsequently was withdrawn in the 1960s when it was discovered that women who took this drug in their first trimester gave birth to children with severe malformations in the limbs and internal organs (7). The return of thalidomide as a therapy in cancers and infections stems from its broad array of anti-inflammatory and anti-angiogenic effects, as demonstrated in diseases such as HIV, Crohn’s disease and leprosy, and cancers such as myeloma (6,8–10).

The cellular target and mechanism of action of thalidomide are poorly understood, but it has been reported in myeloma to suppress angiogenic factors such as vascular endothelial growth factor (VEGF), and inflammatory genes such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (6,8).

Proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (11,12). PPARs heterodimerize with the retinoid X receptor (RXR), and, upon binding to peroxisome proliferator response elements (PPREs) located in the promoter region of target genes, regulate the expression of genes associated with lipid metabolism and adipocyte differentiation. Additionally, PPARs have been shown to regulate inflammatory responses by antagonizing nuclear factor kappa B (NFκB) and activator protein 1 (AP-1) signaling pathways (13–15). To date, three PPAR subtypes have been isolated and named PPARα, PPARβ and PPARγ (12). Each of these subtypes has distinct tissue distribution and is encoded by a separate gene. Of the three subtypes, PPARγ expression has been suggested as a potential marker for lung cancer (16). It has been shown that, upon addition of PPARγ selective agonists to lung cancer cells, growth was inhibited through induction of differentiation and apoptosis (17,18). Additionally, decreased PPARγ expression has been correlated with poor prognosis in patients with lung cancer, suggesting that this gene may be lost as lung cancer progresses (16).

Because LCC metastasizes earlier than other forms of NSCLC, metastasis is an angiogenesis-dependent process, and thalidomide is an anti-angiogenic drug, we evaluated thalidomide as a candidate drug for this solid tumor. We hypothesized that thalidomide would be an effective anticancer...
drug in LCC and that its anticancer properties would be mediated through PPARγ. We used in vitro and in vivo approaches to address the mechanism of action.

Materials and methods

Cell growth studies
NSCLC cell lines (NCI-H1299, NCI-H460, A-549, NCI-H661, NCI-H520, NCI-H596, NCI-H522) were purchased from ATCC (Manassas, VA). Normal bronchial epithelial lung cells were purchased from Cambrex (Walkersville, MD) and grown in BEGM defined medium (Cambrex, Walkersville, MD). NSCLC cell lines were maintained in RPMI media supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5% heat-inactivated FBS (all from Invitrogen, Carlsbad, CA). Thalidomide 1 µg/ml (Calbiochem, San Diego, CA) or vehicle was added daily and complete media changes were performed every 48 h. At the end of 8 days, cell growth was measured by the WST-1 colorimetric assay (Roche, Indianapolis, IN). In this assay, the tetrazolium salt WST-1 is cleaved by mitochondrial dehydrogenases in viable cells producing formazan, whose dark red color can be measured spectrophotometrically at 440 nm. Three independent experiments were conducted and averages and standard error calculated based on these findings. Data are reported as percent inhibition in cell growth, as compared with vehicle-treated controls.

Western analysis
5 x 10^6 NSCLC cells were plated in 6-well dishes (Corning, Corning, NY), and after attachment treated daily with varying doses of thalidomide or vehicle control. After 48 h, whole cell lysates were prepared by scraping cells in lysis buffer (1% SDS, 10 mM Tris pH 7.4, sonicating extracts (level 2, pulse 10 s, rest 5 s; three cycles per sample), and centrifuging (35 min 14,000 r.p.m.) at 4°C. Protein concentration was determined by the Bio-Rad Protein Assay dye, using IgG as a standard (Bio-Rad, Hercules, CA). For each cell line, 60 µg protein was electrophoresed on a 10% Bis-Tris Novex minigel (Invitrogen). After transfer, blots were probed with 1:2000 enhanced chemiluminescence reagents were mixed and transferred to a 96-well plate. Luminescence was measured using a Tropix microplate luminometer. Some wells were blocked for 1 h in 1.5% horse serum and incubated overnight with 1:200 mouse polyclonal PPARγ (ES) antibody (Santa Cruz, CA). Antibody binding was visualized by the avidin-biotin complex technique (Vector Laboratories, Burlingame, CA).

Protein array
1 x 10^5 NCI-H460 or NCI-H1299 cells were plated in 6-well dishes, and after attachment treated with 1 µg/ml thalidomide or vehicle control. For 48 h, 1 ml of supernatant was collected from each dish and a human cytokine protein array (Raybiotech, Norcross, GA) was assayed according to manufacturer’s instructions. Briefly, cytokine membranes were blocked in 1 x blocking buffer for 30 min. After blocking, 1 ml of sample was added to membranes and allowed to incubate for 1.5 h. Membranes were washed and 1 ml of biotin-conjugated anti-cytokine antibody mix was added to membranes, and allowed to incubate for 2 h. After membrane washing, 2 ml of diluted HRP-conjugated streptavidin was added. After a 60-min incubation, followed by washing, detection was performed using enhanced chemiluminescence.

Results

Growth inhibition of NSCLC cells by thalidomide was subtype specific
To study the effect of thalidomide on NSCLC cell growth, cells were treated with 1 µg/ml thalidomide or vehicle control for 8 days. As shown in Figure 1a, after 8 days of treatment, cell proliferation was reduced 40–60% in the three LCC cells that were tested. This is in contrast to the adenocarcinoma cell line, in which the reduction in cell proliferation after 8 days of treatment was much less (Figure 1b). Interestingly, no significant reduction in growth inhibition was observed in the primary normal human bronchial epithelial (NHBE) cells.

Thalidomide significantly increased PPARγ protein expression in all LCC cell lines
To assess the effect of thalidomide on PPARγ protein expression, western analysis was performed on cell lysates from thalidomide-treated LCC cells. In all LCC cells, PPARγ protein expression was lower than in normal primary bronchial epithelial cells. This reduction was specific to the PPARγ1 isoform since PPARγ2 is expressed in normal lung cells. After 48 h of incubation of LCC cells with thalidomide, the PPARγ1 isoform was increased in all LCC cells (Figure 2a), with the highest dose being comparable with those found in the NHBE cells. Densitometry analysis of these cells showed this effect to be dose-dependent (Figure 2b). Thalidomide also induced the PPARγ2 isoform in a dose-dependent fashion. This increase in PPARγ expression was specific to the LCC cell lines, and not
observed in the adeno- and squamous cell carcinoma cells (data not shown).

**Thalidomide significantly increased PPRE reporter activity in LCC cell lines, but not in A549 adenocarcinoma cells**

To assess whether the PPARγ induction by thalidomide had functional consequences, a PPRE reporter assay was performed. We found that in both LCC cell lines tested, thalidomide increased PPRE reporter activity in a dose-dependent manner, with the highest dose (100 μg/ml) yielding 2.5-3 times the activity of TK controls (Figure 3a and b). This highest dose was comparable with the PPARγ selective agonist, troglitazone, which served as a positive control. In sharp contrast, no differences were found in PPRE reporter activity in A549 adenocarcinoma cells transfected in a similar manner (Figure 3c). In this cell line, troglitazone (selective PPARγ agonist) still increased PPRE activity, suggesting that the lack of effect in the thalidomide-treated cells was not due to assay conditions.

**Thalidomide decreased NFκB reporter activity in LCC cells treated with thalidomide, but not in A549 adenocarcinoma cells**

Since PPARγ has been shown to interfere with the anti-apoptotic NFκB signaling pathway, we measured NFκB activity in cells treated with varying doses of thalidomide. For the two LCC cell lines tested (NCI-H460 and NCI-H1299), control cells that received vector (pTAL-NFκB-Luc) but no thalidomide treatment had very high levels of NFκB. When cells were treated with increasing doses of thalidomide for...
24 h, a dose-dependent decrease in NFκB expression was seen, with the highest dose of thalidomide having 2.5--3 times lower NFκB activity (Figure 4a and b). This decrease in NFκB activity was not found in the A549 adenocarcinoma cells (Figure 4c), even though A549 cells treated with 10 mM troglitazone did have a reduction in the NFκB reporter activity.

**Thalidomide increased apoptosis in LCC cell lines, but not in A549 adenocarcinoma cells**

NFκB is a pro-survival factor in cancer cells, and because we found that thalidomide blocks NFκB activity, we tested whether thalidomide could induce apoptosis in LCC cells. Annexin V staining and FACS analysis was performed on cells treated with 1 μg/ml thalidomide (or vehicle) for 24 h. As demonstrated in Figure 5a, NCI-H460 cells underwent a 4-fold increase in apoptosis (8.07 versus 1.74; sum of quadrants B + D) and NCI-H1299 cells had twice as many apoptotic cells in thalidomide treated (10.15) versus control treated (5.67) cells (the sum of quadrants B + D), when treated with thalidomide (Figure 5b). There was no significant difference (P < 0.01) in apoptosis in A549 cells (data not shown).

**Thalidomide treatment of LCC cells decreased inflammatory and angiogenic proteins**

To assess whether thalidomide was an anti-angiogenic factor for lung cancer, a human cytokine protein array was conducted in thalidomide treated versus control cells. NCI-H460 and NCI-H1299 LCC cells were treated with 1 μg/ml thalidomide (or vehicle control) and supernatant collected after 48 h. As seen in Figure 6a, NCI-H460 cells treated with thalidomide had lower growth-related oncogene (GRO), epithelial cell derived-neutrophil activating peptide-78 (ENA-78) and angiotensin levels. In NCI-H1299 cells treated with thalidomide, the angiogenic cytokine IL-8 was significantly lower than in
control cells. No differences were found in other cytokines including GM-CSF, GCSF, I-309, IL-1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 13, 15, IFN-γ, MCP, MCSF, MDCV, MIG, MIP, RANTES, SCF, SDF-1, TARC, TGF-α/β, EDG, IGF-1, OSM, VEGF, PDGF, Tpo and leptin. In both LCC cell lines, cyclooxygenase 2 (COX-2) protein levels were high compared with normal lung cells that did not express COX-2 (data not shown). Thalidomide treatment of LCC cells decreased COX-2 protein levels significantly in a dose-dependent manner (Figure 6b), restoring the levels to the undetected levels found in the NHBE cells.

Intratumoral injection of thalidomide reduced NCI-H1299 tumor growth

To assess whether in vivo thalidomide could decrease lung cancer growth, 24 female nude mice were injected with 1 × 10⁶ NCI-H1299 cells according to the timeline shown in Figure 7. Ten days after injection, tumor formation was seen in all mice and treatments were initiated. As displayed in Figure 8a, there was no difference between vehicle-treated mice and those treated with the lowest dose of thalidomide (2 mg/kg). Mice treated with 20 mg/kg thalidomide had a 20% reduction in tumor growth, and mice treated with the highest dose (200 mg/kg) had growth inhibition of 64% (Figure 8b). No toxicity was found in mice treated with any of the doses of thalidomide (data not shown).

Intratumorally injected thalidomide increased PPARγ protein in mouse tumors

To examine whether mouse tumors had higher immunolabeling of PPARγ protein, immunohistochemistry on excised tumors was performed. At the time of death, mouse tumors were excised, stored in 10% formalin, paraffin-embedded and sectioned (5 μm sections) for immunolabeling of PPARγ protein. As shown in Figure 9, there is a large increase in PPARγ protein expression in the 200 mg/kg thalidomide-treated mice compared with control tumors that had almost no PPARγ expression. Two sections from two different animals are displayed.

Discussion

Despite recent advances in novel chemotherapeutic agents for the treatment of NSCLC, as well as in the understanding of the molecular basis of this disease, the number of deaths due to lung cancer is still greater than for any other form of cancer (1). The very poor (10–15%) 5-year survival rate is primarily due to a high rate of metastasis. Of the four subtypes of NSCLC, LCC has been shown to follow an extremely aggressive clinical course, growing and metastasizing earlier than other NSCLC histological subtypes (2). Since the growth and metastasis of a neoplasm is dependent on adequate vascular support to sustain proliferation, survival and spread of the malignant cells, tumor growth and metastasis are considered to be angiogenesis-dependent. Therefore, therapeutic strategies aimed at inhibiting angiogenesis are theoretically attractive.

Thalidomide is an anti-angiogenic and anti-inflammatory drug that has proven effective in various inflammatory diseases and cancers, and is beginning to be evaluated as an agent against the growth of solid tumors. However, to date, the mechanism of action by which thalidomide exerts its effects is poorly understood. Some have suggested that thalidomide exerts its effects through the down-regulation of
tumor-released signaling molecules that stimulate angiogenesis. These molecules include IL-6, bFGF, VEGF and TNF-α (5,19). Others believe thalidomide increases cell adhesion molecules (20), suppresses COX-2 activity (21) or alters cytokine expression (22). In this manuscript, we report that thalidomide inhibition of growth does not affect all NSCLC cells but is rather specific to LCC cells. Indeed, whereas LCC cells were largely inhibited after 8 days of treatment, adeno-, adenosquamous and squamous cell carcinomas were only mildly affected. At the molecular level, we focused on PPARγ, a member of the nuclear hormone receptor superfamily whose expression is abundant in normal lung tissue, but often lacking in lung cancer tissues (16).

It has been documented previously in lung cancer cells that PPARγ-selective agonists such as ciglitazone and PGJ2 can inhibit growth of NSCLC cells through the induction of apoptosis, promotion of differentiation and the down-regulation of cell cycle proteins such as Cyclin D1 (17,18). Because we have seen that the PPARγ-selective agonist troglitazone is also effective at inhibiting growth of NSCLC cells, and morphologically seems to differentiate the cells, we used this as...
a positive control in our studies. PPARγ is present in two isoforms, PPARγ1 and PPARγ2, resulting from alternate promoter usage. Structurally, PPARγ2 contains an additional 30 aa at the N-terminal end relative to PPARγ1. Each of these two isoforms has specific function and tissue distribution. PPARγ1 is expressed in many tissues, but the expression of PPARγ2 is limited exclusively to adipose tissue where it has been shown to play a key role in adipogenesis (23). In our study we found that PPARγ1 expression was lower in LCC cells than in primary NHBE cells. Moreover, thalidomide increased the level of this isoform in a dose-dependent manner in LCC cells, with the highest dose increasing the level of expression to that of the NHBE cells (Figure 2a). This PPARγ was functional, as demonstrated through a PPRE reporter assay (Figure 3). As expected, the primary NHBE cells do not express PPARγ2, but the expression of this isoform can be forced upon thalidomide treatment. Others have suggested that forced expression of either PPARγ1 or PPARγ2 promotes differentiation along the adipocyte lineage (23) and this differentiation seems to be found in responding tumor cells (24). Although we did not measure adipocyte-specific genes and the accumulation of lipid, it is possible that our tumor cells are being differentiated toward this phenotype. Unlike the LCC cells that lose their expression of PPARγ compared with NHBE cells, the adenocarcinoma cells normally contain measurable amounts of both PPARγ1 and PPARγ2 (data not shown); this, possibly explains why these cells are not as responsive to thalidomide treatment.

Consistent with the fact that PPARγ blocks inflammatory responses by antagonizing NFκB and AP-1 signaling pathways (13–15), we found that the induction of PPARγ expression due to thalidomide treatment was correlated with a dose-dependent inhibition in NFκB activity (Figure 4). In contrast, untreated LCC cell lines had very high NFκB activity due to the inflammatory response that accompanies this cancer (Figure 4), agreeing with previously reported literature (25). Since it is well known that NFκB activation is a pro-survival phenomenon used by cancer cells to avoid apoptosis, we studied whether the addition of thalidomide increases apoptosis in our LCC cells. We found that in both cell lines, thalidomide treatment could induce apoptosis (Figure 5), although the level of apoptosis induction is probably not great enough to account for the total growth inhibition that we have found in the LCC cells. Thalidomide has been shown in hematologic diseases, such as myeloma, to decrease neoplastic cell growth by altering some of the pro-inflammatory cytokines such as TNF and IL-6. Whereas we failed to detect differences in pro-inflammatory cytokines upon thalidomide treatment (Figure 6a), we did find that one angiogenic cytokine (IL-8) in the NCI-H1299 LCC cell line, and three angiogenic cytokines in the NCI-H460 cells (Ang, GRO and ENA-78) were down-regulated in thalidomide treated cells. This is important because ENA-78 and IL-8 have both been found to be elevated in NSCLC, consistent with the high vascularity of this cancer, and neutralizing ENA-78 and IL-8 antibodies have been shown to decrease tumor growth, vascularity and spontaneous metastasis (26,27). Additionally, we did find that thalidomide down-regulated the strong COX-2 expression that accompanies NSCLC (Figure 6b). This is noteworthy because COX-2 is believed to be involved in the invasiveness of NSCLC, as well as in resistance to apoptosis (28). Moreover, over-expression of COX-2 in NSCLC has been linked to poor prognosis for this disease (28). It has been documented that COX-2 modulates production of angiogenic factors in colon cancer cells (29), and therefore blocking this inflammatory agent may prevent angiogenesis and stimulate immune surveillance and apoptosis. In macrophages (30), thalidomide can decrease the expression of COX-2. Our data support that this is also true for LCC cells.

In our in vivo experimentation, we found that intratumoral injection of thalidomide (using the highest concentration tested) resulted in a 64% reduction in tumor growth (Figure 8), accompanied by decreased vascularity (observation). To confirm that some of this growth inhibition may be correlated with an elevated level of PPARγ, immunohistochemical analysis of PPARγ was performed on excised tumors. In control tumors, we barely found any detectable levels of PPARγ (Figure 9). In contrast, thalidomide-treated tumors showed significantly more staining than control tumors, which correlates well with our in vitro western data (Figure 2). As a control, we have tested normal bronchial tissue and found a significant number of PPARγ-positive cells, mainly in luminal position (data not shown), suggesting that the induction of PPARγ by thalidomide may be restoring the normal phenotype.

This work implicates thalidomide as being involved in the PPARγ pathway, and suggests that thalidomide mediates some of the effects of PPARγ in LCC cells. To our knowledge, this is the first report of a link between thalidomide and PPARγ. Whether thalidomide acts as a direct ligand for PPARγ or indirectly through other proteins has yet to be elucidated. Thus, this work may further help to identify one possible mechanism by which thalidomide exerts its anticancer effects, as well as define new molecular targets in the treatment of lung cancer.
Supplementary material

Supplementary material can be found at: http://www.carcin.oupjournals.org/.

Acknowledgements

We thank Barbara Taylor for her assistance with the flow cytometry and Dr R.M. Evans for the kind gift of the PPRE3-TK-LUC and TK-LUC reporter constructs.

References


Received November 10, 2003; revised June 1, 2004; accepted June 6, 2004