Acting via a Cell Surface Receptor, Thyroid Hormone Is a Growth Factor for Glioma Cells

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Abstract
Recent evidence suggests that the thyroid hormone L-thyroxine (T4) stimulates growth of cancer cells via a plasma membrane receptor on integrin αVβ3. The contribution of this recently described receptor for thyroid hormone and receptor-based stimulation of cellular mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase 1/2 (ERK1/2)] activity, to enhancement of cell proliferation by thyroid hormone was quantitated functionally and by immunologic means in three glioma cell lines exposed to T4. At concentrations of 1 to 100 nmol/L, T4 caused proliferation of C6, F98, and GL261 cells, measured by accumulation of proliferating cell nuclear antigen (PCNA) or the cellular uptake of tritiated thymidine (14). This latter effect of T4 is reproduced by stimulation of growth factors, such as basic fibroblast growth factor (FGF), thyroid hormone–binding site on integrin αVβ3 is at or near the RGD recognition site and that an RGD peptide will displace thyroid hormone from integrin and will block the cellular actions of the hormone (1). An interesting feature of this hormone receptor is that tetraiodothyroacetic acid, a hormone analogue with reduced thyromimetic activity (4), competes with T4 at the receptor site (1).

Glioblastoma, the most common primary brain tumor in adults (5, 6), carries a poor prognosis despite a variety of therapeutic approaches. The astrocyte is the cell of origin in glioblastomas, and astrocytes have been shown previously to be a focus of thyroid hormone action (7–9). Modulation of the state of actin (7, 10) and integrin (9), have been reported. By stimulation of growth factors, such as basic fibroblast growth factor (FGF), thyroid hormone may stimulate neuronal cell proliferation (11). Hercbergs et al. (12) have reported in 2003 that glioblastoma patients rendered mildly hypothyroid by thioamide treatment exhibit significantly improved duration of survival, raising the possibility that endogenous thyroid hormone may be a growth factor for glioblastoma.

Other neoplasms may also be subject to the growth-promoting activity of thyroid hormone. Cristofanilli et al. (13) showed in 2005 that spontaneous clinical hypothyroidism may decrease the aggressiveness of breast cancer and reduce the incidence of the tumor. We have reported that the response of MCF-7 breast cancer cells to treatment with a physiologic concentration of T4 is reproduced by exposure of cells to T4-agarose, an immobilized form of the hormone that cannot enter the cell (14), implicating a cell surface receptor site in this trophic effect of thyroid hormone on breast cancer cells.

With this evidence for a cell surface receptor site as the initial step in the trophic effect of thyroid hormone on breast cancer cells and the clinical evidence supporting a role for thyroid hormone in the growth of glioblastoma, we have examined the possibility that iodothyronines, acting at the cell surface, are growth factors for a rat glioma (C6) cell line as well as other glial tumor models.

Introduction
We have described recently a specific cell surface receptor for the thyroid hormone L-thyroxine (T4) on integrin αVβ3 that is linked to the activation of hormone of mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase 1/2 (ERK1/2)] and, downstream of MAPK, to complex transcriptional events, such as angiogenesis (1). The integrins are structural plasma membrane proteins whose extracellular domains bind to matrix and other proteins and whose intracellular domains have been shown by others to activate MAPK (2). Integrin αVβ3 contains an RGD (arginine-glycine-aspartate) recognition site that is essential to the interactions of the integrin with its extracellular matrix (ECM) protein ligands (2, 3). We have presented evidence that the thyroid hormone–binding site on integrin αVβ3 is at or near the RGD recognition site and that an RGD peptide will displace thyroid hormone from integrin and will block the cellular actions of the hormone (1). An interesting feature of this hormone receptor is that tetraiodothyroacetic acid, a hormone analogue with reduced thyromimetic activity (4), competes with T4 at the receptor site (1).

Materials and Methods

Cell lines. The rat glioma cell line C6 was purchased from American Type Culture Collection (Rockville, MD), whereas F98 and GL261 cells were
Concentrations of 1 to 100 nmol/L over a 24-hour period. This effect was the accumulation of PCNA in C6, F98, and GL261 cells at total hormone concentrations from 1 to 100 nmol/L, over a 24-hour period. This effect was T4 concentration dependent. Representative of three experiments with each cell line. The GL261 cells showed some PCNA antigen in the absence of hormone, and hormone concentration-dependent PCNA was further increased by T4, as shown in the representative immunoblot and graph derived from results of three experiments. B, in combined results from three studies of radiolabeled thymidine incorporation, a similar dose-response effect of T4 (1-100 nmol/L) is seen in C6 cells.

Cell fractionation. Cell fractionation and preparation of nucleoproteins were done as in previously reported methods (15). Nuclear extracts were prepared by resuspension of the crude nuclei in high-salt buffer (hypotonic buffer with 420 mmol/L NaCl and 20% glycerol) at 4°C for 1 hour. The supernatants were collected after subsequent centrifugation at 4°C and 13,000 rpm for 10 minutes.

Immunoblotting. The techniques have been standardized in our laboratory (15–17). Nucleoproteins were separated on discontinuous SDS-PAGE and then transferred by electrophoretic transfer to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% skim milk in TBS containing 0.1% Tween 20, the membranes were incubated with various antibodies overnight. Secondary antibodies were either goat anti-rabbit IgG (1:1000, DAKO) or rabbit anti-mouse IgG (1:1000, DAKO), depending on the origin of the primary antibody. Immunoreactive proteins were detected by chemiluminescence, and blots were quantitated densitometrically. All experiments were carried out at least three times with normal protocols to a value of 1 in control samples.

Thyroid hormone causes glioma cell proliferation. We have shown recently that T4 induces expression of PCNA in human breast cancer cells (14). A similar response to T4 was seen in three different glioma cell lines, as shown by representative studies seen in Fig. 1A. C6, F98, and GL261 cells were treated for 24 hours with T4, and a dose-response effect was evident with hormone concentrations from 1 to 100 nmol/L. In addition, radiolabeled thymidine incorporation studies were used to characterize this hormone effect. A concentration-dependent response to T4, comparable with that seen in PCNA expression studies, is seen in a representative thymidine uptake study in C6 cells, with the greatest effect seen at a physiologic hormone concentration of 100 nmol/L (Fig. 1B).

Available in the laboratory of author R.A.F. C6 cells were maintained in F12K medium supplemented with 10% fetal bovine serum (FBS). F98 and GL261 cells were maintained in DMEM supplemented with 10% FBS. All cell cultures were cultured in a 5% CO2/95% air incubator at 37°C. Before treatment, cells were exposed to 0.25% hormone-stripped FBS-containing medium for 2 days (14).

Reagents and antibodies. T4, T3, tetraiodothyroacetic acid, RGD peptide, and phorbol 12-myristate 13-acetate (PMA) were obtained from Calbiochem (La Jolla, CA). CGP41251, a protein kinase C (PKC) inhibitor, was purchased from Calbiochem (La Jolla, CA). PD98059, a MAPK kinase (MAPK/ERK kinase (MEK)) inhibitor, was purchased from Calbiochem (La Jolla, CA), and RGD peptide was from Sigma-Aldrich Corp. (St. Louis, MO). Polyclonal rabbit anti-phosphorylated MAPK (anti-pERK1/pERK2) was purchased from Cell Signaling (Beverly, MA), and monoclonal mouse anti-PCNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG and rabbit antimouse IgG were purchased from Dako (Carpinteria, CA). Chemiluminescence reagent (enhanced chemiluminescence) was from Amersham (Piscataway, NJ). PD98059, a MAPK kinase [MAPK/ERK kinase (MEK)] inhibitor, was purchased from Calbiochem (La Jolla, CA), and RGD peptide was from Sigma-Aldrich. CPG41251, a protein kinase C (PKC) inhibitor, was kindly provided by Ciba-Geigy (Basel, Switzerland).

Figure 1. L-Thyroxine (T4) stimulates accumulation of PCNA and uptake of radiolabeled thymidine in C6, F98, and GL261 glioma cells. A, T4 stimulated accumulation of PCNA in C6, F98, and GL261 cells at total hormone concentrations of 1 to 100 nmol/L, over a 24-hour period. This effect was T4 concentration dependent. Representative of three experiments with each cell line. The GL261 cells showed some PCNA antigen in the absence of hormone, and hormone concentration-dependent PCNA was further increased by T4, as shown in the representative immunoblot and graph derived from results of three experiments. B, in combined results from three studies of radiolabeled thymidine incorporation, a similar dose-response effect of T4 (1-100 nmol/L) is seen in C6 cells.

Figure 2. Pretreatment with RGD peptide or tetraiodothyroacetic acid before addition of T4 results in inhibition of the hormone effect on PCNA expression in C6 cells. Cells pretreated with RGD peptide (5-500 nmol/L) or tetraiodothyroacetic acid (tetrac) (100 nmol/L) for 30 minutes showed inhibition of T4-stimulated cellular PCNA expression. The response to RGD peptide was dose dependent. Neither the peptide nor tetraiodothyroacetic acid alone significantly affected PCNA expression. Top, representative study; bottom, results of three similar experiments.
T4 stimulation of C6 cell proliferation requires hormone interaction with its plasma membrane receptor, integrin \( \alpha_{v}\beta_{3} \).

As indicated above, several nongenomic actions of T4, effects that do not require primary interaction of the hormone with the nuclear thyroid hormone receptor, TR\( ^{\beta} \), are initiated at a plasma membrane receptor on integrin \( \alpha_{v}\beta_{3} \). Such actions can be blocked with simultaneous administration of either the T4 analogue tetrac (tetraiodothyroacetic acid) or an RGD peptide, both of which inhibit hormone binding to the integrin receptor (1). C6 cells were therefore incubated with T4 in the presence or absence of these inhibitors (Fig. 2). Increasing concentrations of the RGD peptide (5-500 nmol/L) progressively inhibited the hormone effect on PCNA expression, shown in both representative immunoblot above and the graph below, which summarizes the results of three experiments. Tetraiodothyroacetic acid alone had no effect on PCNA expression but blocked the proliferative effect of T4 in C6 cells, consistent with inhibition of T4 binding to integrin \( \alpha_{v}\beta_{3} \) (1).

\( \text{L-Thyroxine induces MAPK (ERK1/2) activation in glial cells, an effect requiring conventional PKC activation and blocked by RGD peptide and tetraiodothyroacetic acid.} \)

On interaction with the plasma membrane integrin receptor, T4 stimulates the MAPK pathway, resulting in phosphorylation and nuclear translocation of ERK1/2 (1, 15). This effect may take place in the presence (16-18) or absence (1, 15, 18) of the nuclear thyroid hormone receptor, TR\( ^{\beta} \), and is therefore independent of TR\( ^{\beta} \). Rat C6 glioma cells were treated with a physiologic total concentration of T4 (100 nmol/L total or <0.1 nmol/L free T4; ref. 1), in the presence or absence of integrin \( \alpha_{v}\beta_{3} \) RGD peptide (5-500 nmol/L). This peptide has been shown to block interaction of protein ligands with the integrin (2, 3), as well as the interaction of \( \text{l-thyroxine with the integrin (1).} \) T4 caused nuclear accumulation of phosphorylated MAPK (pERK1/2) in C6 cell nuclei (Fig. 3A), indicating kinase activation and consequent translocation to the nucleus. This thyroid hormone-induced MAPK activation was inhibited by the RGD peptide in a peptide concentration-dependent manner (Fig. 3A). The RGD peptide, alone, did not activate MAPK. Tetraiodothyroacetic acid, which inhibits binding of T4 to the integrin receptor, did not cause MAPK activation but inhibited the activation of MAPK by T4 (Fig. 3B). Similar studies were conducted in F98 glioma cells and are seen in Fig. 3C. Again, T4 stimulation of ERK1/2 phosphorylation and appearance of PCNA were both suppressed by tetraiodothyroacetic acid. These studies were also conducted in GL261 glioma cells, and Fig. 3D shows inhibition by tetraiodothyroacetic acid of both MAPK activation and cell proliferation induced by T4.

Treatment of C6 cells with T4 in the presence of the MEK (MAPK kinase) inhibitor, PD98059, produced an expected loss of hormone action on nuclear accumulation of phosphorylated ERK1/2 (Fig. 4A). PD98059 in the absence of thyroid hormone had no effect on activation of MAPK. We have shown previously that activation of MAPK by T4 requires activity of a conventional PKC (cPKC; ref. 15). The contribution of cPKC to T4-induced MAPK activation was shown in C6 cells; such activation was blocked by the cPKC inhibitor, CgP41251, in a dose-dependent manner (Fig. 4A). Further, depletion of cellular PKC by

\[ \text{Figure 3. RGD peptide and tetraiodothyroacetic acid inhibit T4 stimulation of MAPK activation and cell proliferation in glioma cells.} \]

A, The RGD peptide alone did not significantly stimulate activation of MAPK in C6 cells. T4 (100 nmol/L) promoted activation of MAPK, as shown by nuclear accumulation of phosphorylated ERK1/2. This latter effect was progressively suppressed by increasing concentrations of the RGD peptide (5-500 nmol/L). Top, representative immunoblot; bottom, normalized results of three similar experiments. B, T4-stimulated MAPK activation in C6 cells was also suppressed by coincubation of T4 (100 nmol/L) with tetraiodothyroacetic acid (tetrac) (100 nmol/L), a finding that we have reported previously in other cell lines (15). Bottom, results of three similar experiments. C, In F98 cells, T4-stimulated MAPK activation and enhanced PCNA accumulation were inhibited by the addition of tetraiodothyroacetic acid. Bottom, normalized results of three similar PCNA studies. D, In GL261 cells, MAPK activation and accumulation of PCNA, stimulated by T4, were both decreased by the addition of tetraiodothyroacetic acid to the hormone incubation. Top, representative immunoblots of three similar experiments; bottom, results of three PCNA experiments.
pretreatment of cells with phorbol ester (PMA) for 24 hours eliminated the effect of hormone on MAPK activation (Fig. 4B). In contrast, short-term treatment of cells with PMA resulted in activation of MAPK (pERK1/2) in the absence of T4, and addition of hormone caused no significant further increase in MAPK activation (Fig. 4B). The effects of MAPK and cPKC inhibition on T4-induced accumulation of PCNA in C6 cells are seen in Fig. 4C. The hormone effect was progressively inhibited by increasing concentrations of either PD98059 or CGP41251, indicating dependence of the effect on cPKC activation and stimulation of the MAPK activation pathway.

I-L3 also stimulates glioma cell proliferation and thymidine incorporation. Parallel studies were conducted with T3, the traditional ligand of the nuclear thyroid hormone receptor, TRβ1. This thyroid hormone also stimulated proliferation of C6, F98, and GL261 cells (Fig. 5A), although the concentrations needed for this effect were supraphysiologic, except with GL261 cells, which expressed PCNA without hormone treatment. We have reported that the affinity of αvβ3 for T3 is substantially lower than the affinity for T4 (1). C6 cell proliferation with T3 treatment was also measured by thymidine incorporation, and results again showed a concentration-dependent hormone response comparable with the results of PCNA studies (Fig. 5B).

Discussion

We have shown recently that a binding site for thyroid hormone exists on the cell surface at an integrin (αvβ3) that is linked to MAPK activity (1), angiogenesis (1), and cell migration (10). The existence of an initiation site for these so-called nongenomic actions of the hormone actions that do not primarily require concentrations of either PD98059 or CGP41251, indicating dependence of the effect on cPKC activation and stimulation of the MAPK activation pathway.

Figure 4. T4 stimulation of MAPK activation in C6 cells is dependent on cPKC activity. A, T4 treatment of C6 cells resulted in activation of MAPK (formation and nuclear translocation of pERK1/2). PD98059 (PD; 3-30 μmol/L) inhibited ERK1/2 phosphorylation, as expected. The cPKC inhibitor, CGP41251 (CGP; 10-100 nmol/L) also suppressed the T4 effect in a dose-dependent manner, as we have reported previously (15). Top, representative immunoblot; bottom, results of three similar experiments. B, MAPK activation in C6 cells was stimulated by T4, but this effect was blocked by PMA treatment (100 ng/mL for 24 hours). Short-term PMA treatment (30 minutes) did not affect hormone action (results not shown). Top, representative immunoblot; bottom, results of three experiments. C, C6 cell PCNA was measured in the presence or absence of T4 and/or PD98059 or CGP41251. Hormone-stimulated PCNA accumulation was progressively inhibited by increasing concentrations of either inhibitor, indicating dependence of the hormone effect on both cPKC and ERK1/2 activation pathways. Top, representative immunoblot of three experiments.

Figure 5. T3 stimulates accumulation of PCNA in glioma cells. A, PCNA accumulation was seen in C6, F98, and GL261 cells treated with 3,5,3'-triiodo-L-thyronine (T3), 1 to 1,000 nmol/L. In C6 and F98 cells, this occurred with T3 concentrations which are supraphysiologic (≥1 nmol/L). As also seen in Figs. 1A and 3D, the GL261 cells expressed PCNA even in the absence of hormone. B, radiolabeled thymidine incorporation was measured in C6 cells treated with 0.1 to 1,000 nmol/L T3.
intracellular binding of thyroid hormone to its nuclear receptor mediated by an integrin caused us to speculate that glioma cell growth in response to thyroid hormone might in fact occur via integrin αvβ3. The observations reported in the present article implicate this integrin in promotion by thyroid hormone of cell growth of glioma cells. The location of the thyroid hormone–binding site is at or near the RGD recognition site on the integrin, as shown elsewhere by the ability of RGD peptide to competitively displace T4 from the integrin (1). We show here, via inhibition or depletion of PKC upstream of MAPK or via inhibition of the MAPK (ERK1/2) pathway at MEK, that the integrin mediates the activation of this pathway and, downstream of MAPK, the action of ERK1/2 on cell division, when the latter was measured by PCNA accumulation or [3H]-thymidine incorporation. RGD peptide and tetraiodothyroacetic acid both blocked the cell growth-promoting activity of thyroid hormone, consistent with initiation of the thyroid hormone effect at the cell surface receptor for thyroid hormone that we have described (1).

Promotion of glioma cell proliferation (19, 20) and differentiation (21) by thyroid hormone in vitro has been described by several laboratories. The hormone also affects myelin gene expression and survival of developing oligodendrocytes (22). In addition, thyroid hormone conditions the relationship of glial cells with ECM proteins, such as laminin (9, 23) and fibronectin (23). Differentiation that is caused by iodothyronines is p21 dependent (20), and the cell-ECM relationship is, at least in part, a function of the action of thyroid hormone on cellular release of FGF (11). The molecular basis for initiation of these actions of thyroid hormone has not been fully defined; however, we have reported previously that iodothyronines can induce FGF2 secretion in endothelial cells by a mechanism that is MAPK (ERK1/2) dependent (24). We have observed a secondary increase in MAPK activation at 24 hours in C6 cells exposed to thyroid hormone (results not shown), which may in fact reflect increased FGF2 secretion, as we have proposed in the proangiogenic action of thyroid hormone (24).

Clinical observations by Hercbergs et al. (12) suggest that the growth of glioblastoma multiforme is thyroid hormone dependent. These authors induced mild chemical hypothyroidism by interfering with thyroid hormonogenesis through administration of propylthiouracil and obtained a 3-fold increase in duration of survival of patients. This experience in the United States has been reproduced in Israel by a group with which Hercbergs is associated (25). Thyroid hormone is known to cross the blood-brain barrier (26, 27), and as noted above, glial cells are nervous system target organ of thyroid hormone action (10, 28). Glial cell migration and, by extension, glial tumor cell migration would be facilitated by ambient levels of thyroid hormone that act to maintain actin in the F-state (28) and that modulate astrocyte integrin-ECM protein interactions (9). Fostering of angiogenesis by thyroid hormone (1, 24) is an adjunctive factor that would also serve to support tumor growth.

The present in vitro observations provide a cellular mechanism by which thyroid hormone can be a growth factor for glioma and the rationale for the clinical observations that reducing ambient (i.e., physiologic) levels of thyroid hormone may improve duration of survival in glioblastoma patients. Because our in vitro observations indicate that the tumor cell growth-promoting activity of thyroid hormone begins at the integrin receptor for the hormone; however, treatment strategies may exist that are alternatives to induction of clinical hypothyroidism. For example, tetraiodothyroacetic acid may be used systemically to block endogenous thyroid hormone–binding to the integrin; tetraiodothyroacetic acid has low-grade thyromimetic activity, except for competition with T4 and T3 at the cell surface receptor site. Proprietary small molecules are also available in our laboratory that mimic the RGD peptide sequence, and we have shown that such molecules can interfere with actions of thyroid hormone that are dependent on the integrin receptor site (29). Use of either of these strategies would permit actions of endogenous thyroid hormone, specifically T3, on mitochondrial respiration to persist, as well as genomic actions of the hormone that are mediated directly by liganding to the nuclear thyroid hormone receptor.

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