

Incomplete Success of Angioinhibitor Therapy in Cancer: Estimation of Contribution of Pro-angiogenic Activity of Patient Thyroid Hormone

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Abstract

Despite the obvious promise of the strategy, pharmaceutical angioinhibition has had variable success in clinical cancer management. Thyroid hormone is a potent pro-angiogenic factor. Endogenous circulating levels of pro-angiogenic thyroid hormone in cancer patients treated with anti-angiogenic drugs may contribute to host resistance to angioinhibition and explain, at least in part, the variable cancer chemotherapeutic responses obtained with anti-angiogenic agents. The chick chorioallantoic membrane (CAM) angiogenesis assay accepts human tumor xenografts and is a system in which individual patient blood samples can be tested in xenograft vasculature for anti-angiogenic content—including thyroid hormone—in the presence of angioinhibitory drug dose escalation. The assay may also be used to screen individual patient tumor biopsy xenografts for susceptibility to angioinhibition.

Keywords: Thyroxine; Angiogenesis; Chick chorioallantoic membrane (CAM) assay; Integrin $\alpha\beta 3$

Introduction

Angiogenesis inhibitor therapy directed at one or several vascular growth factors or their receptors in cancer has had variable success, despite the hypervascularity of many tumors. Treatment success that is only partial may reflect redundancy of vascular growth factors in cancer cells, tolerance for hypovascularity and hypoxia of certain tumors, resistance mutations in growth factor receptor genes or the presence of effective circulating endogenous pro-angiogenic substances in the cancer patient. Estrogen and progesterone are examples of steroids that are pro-angiogenic in their target tissues [1,2] and target tissue cancers.

By multiple molecular mechanisms, thyroid hormone—L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyronine (T_3) [3,4]—and other thyroid hormone agonist analogues [5,6] have been shown to be pro-angiogenic. Pharmacologic elimination of this action of thyroid hormone at a cell surface receptor for the hormone can in days reduce the vascularity of human tumor xenografts by 40-50% [7-9] and contribute to shrinkage of tumor volume. Thyroid hormone can also support proliferation of cancer cells [10-12], so that change in tumor size reflects several mechanisms when activity of the hormone is reduced at the tumor site. The pro-angiogenic activity of T_4 and T_3 is initiated at a cell surface receptor for the hormones on a structural protein of the plasma membrane, integrin $\alpha\beta 3$ [13]. The original description of this receptor was in an angiogenic model system, the chick chorioallantoic membrane (CAM) assay [13]. The integrin has multiple functions that relate to extracellular matrix proteins [14], to growth factor receptors on the cell surface [15] and, within the cell, to cell structure [16] and specific gene transcription [17]. The existence of the iodothyronine receptor on the integrin has broadened our appreciation of the contributions that a small molecule ligand-integrin interaction can make to regulation of cancer-relevant angiogenesis [18,19] and to gene transcription involved in cancer cell survival pathways [8,19,20]. This iodothyronine receptor is subject to specific blockade with tetraiodothyroacetic acid (tetrac), a derivative of T_4 , and a nanoparticulate covalent conjugate of tetrac [19]. These two agents

rapidly express in tumor xenografts the loss of vascularity to which we referred [7-9].

That cancer in the setting of hypothyroidism, spontaneous or induced, behaves differently than in euthyroidism is suggested by clinical studies of glioblastoma [21], breast cancer [22] and, apparently, of renal cell carcinoma [23-25]. In each of these settings, desirable clinical behavior accompanies decreased circulating levels of thyroid hormone and this may in part reflect actions of thyroid hormone on angiogenesis. It is important to emphasize that clinical hypothyroidism is not advocated here to affect tumor cell behavior desirably, since medically-induced subclinical hypothyroidism (asymptomatic elevation of serum thyrotropin [TSH]) has been effective in importantly arresting progression and increasing overall survival in glioblastoma patients [21]. In a second cancer-relevant context, it should also be pointed out that thyroid hormone is anti-apoptotic [26,27], supporting survival of the cancer cell and its angiogenic activity. The hormone has been shown to inhibit apoptosis induced *in vitro* by specific agents such as resveratrol [27] and ceramide (HY Lin: unpublished).

We propose that certain host circulating nonpeptide hormones—the model here is thyroid hormone—that are pro-angiogenic may limit in selected patients or specific clinical settings the effectiveness of anticancer therapy that is focused exclusively on angiogenesis.

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Estrogen [28,29], as suggested above, and gonadotropins [30] are other possible examples of nonpeptide and peptide hormones, respectively, that may modulate tumor-relevant angiogenesis. In the case of thyroid hormone, elevated circulating free T_4 (FT_4) may be encountered transiently or longer in the nonthyroidal illness (NTI) syndrome or in patients receiving high-dose L- T_4 replacement. The NTI syndrome includes low serum T_3 concentration and suppressed TSH [31]. Thus, in the setting of NTI, reduced circulating levels of T_3 and TSH are not germane to angiogenesis, but FT_4 is supportive. The issue may also be raised for consideration that endogenous free T_4 concentrations in the upper quartile of the reference range in euthyroid patients may support angiogenesis. In the state of subclinical hypothyroidism, FT_4 is normal and circulating TSH, another peptide hormone, is by definition elevated and conceivably could be a pro-angiogenic factor in nonthyroidal tumor tissue [32]. The regulatory input of thyroid hormone into angiogenesis is discussed in more detail below.

The question is how to estimate the contribution of any endogenous host growth factors to suboptimal efficacy of angioinhibitor therapy. The CAM assay accepts human cancer xenografts in which tumor growth properties are readily detected [12] and tumor vascularity quantitated [4,13]. This assay is accessible and inexpensive. It is a vehicle in which responsiveness of standard tumor cell lines to anti-angiogenic treatment agents has been assessed. As discussed below, the assay is a possible test system in which individual patients' plasma or serum may be screened for pro-angiogenic properties in the presence of xenografted tumor and specific anti-angiogenic biological or chemical agents.

Pro-angiogenic Properties of Thyroid Hormone

Studies in myocardium by Tomanek et al. [3,33] established that T_4 and a thyroid hormone analogue, diiodothyropropionic acid (DITPA) [34], stimulated coronary arteriolar growth. Subsequently and prior to the description of the existence of the thyroid hormone receptor on $\alpha v\beta 3$, we showed that the hormone was pro-angiogenic in the CAM model [4] by a cell surface-based mechanism. Components of the mechanism of angiogenesis included transcription of the basic fibroblast growth factor (*bFGF*) gene and release of the gene product into the medium of the CAM. Thyroid hormone also induces microtubule formation by human dermal microvascular endothelial cells [5] and stimulates migration of endothelial cells towards a vitronectin cue in the modified Boyden chamber apparatus (SA Mousa: unpublished observations). Evidence subsequently developed in the CAM assay suggested that the hormone may also influence the activities of vascular endothelial growth factor (VEGF) [35] and platelet-derived growth factor (PDGF) (SA Mousa: unpublished). That is, a pharmacologic inhibitor of thyroid hormone action at $\alpha v\beta 3$ decreased pro-angiogenic actions of VEGF and PDGF in the CAM. The mechanism is thought to involve crosstalk between integrin $\alpha v\beta 3$ and the adjacent receptors for VEGF, PDGF and bFGF on the cell surface. It is also clear that thyroid hormone can modulate certain non-angiogenic functions of the plasma membrane receptors for epidermal growth factor (EGF) [36] and insulin-like growth factor-1 (IGF-1) [37]. But EGF and IGF-1 [38,39] do have pro-angiogenic properties and it will be important to determine whether crosstalk between receptors for these growth factors and the iodothyronine receptor on $\alpha v\beta 3$ exists. The complexity of the relationship between iodothyronines and IGF-1 and the latter's receptor (IGF1R) is demonstrated in studies of rodent skeletal myoblasts. In these cells, thyroid hormone and IGF-1 individually stimulate muscle cell proliferation and hexose uptake, but the hormone

blocks action of IGF-1 on both functions [37]. Thus, thorough studies of these agents, individually and together, are required in a standard angiogenesis model, such as the CAM, to determine whether these growth factors work to block therapeutic angioinhibition.

We have confirmed that certain agonist thyroid hormone analogues such as DITPA [6] and the non-iodinated synthetic hormone analogue, GC-1 [40], are pro-angiogenic in the CAM assay. DITPA had previously been shown to increase myocardial VEGF and bFGF in the nonischemic rat heart [34]. Pro-angiogenic qualities of thyroid hormone have also been shown experimentally in the ischemic limb [41] and in wound-healing [42,43] (Figure 1).

Thyroid Hormone Analogues and Angiogenesis Associated with Cancer Xenografts

In xenografts in the euthyroid nude mouse of human renal cell carcinoma [7], follicular thyroid cancer [44], medullary carcinoma of the thyroid [8] and pancreatic cancer [9], we have induced frank and rapid decrease in vascularity of the grafts with a pharmacologic inhibitor of actions of T_4 and T_3 at their receptor on $\alpha v\beta 3$. This angiogenesis inhibitor is unmodified or nanoparticulate tetrac [19]. The latter involves covalent binding of tetrac to a nanoparticle to prevent cellular uptake of the thyroid hormone analogue and limit drug activity to the extracellular domain of the integrin $\alpha v\beta 3$. The pro-angiogenic activity of thyroid hormone in the CAM assay can also be blocked with monoclonal antibody to $\alpha v\beta 3$ [19].

It should be emphasized that integrin $\alpha v\beta 3$ is expressed generously by rapidly-dividing blood vessel cells and by cancer cells. Quiescent

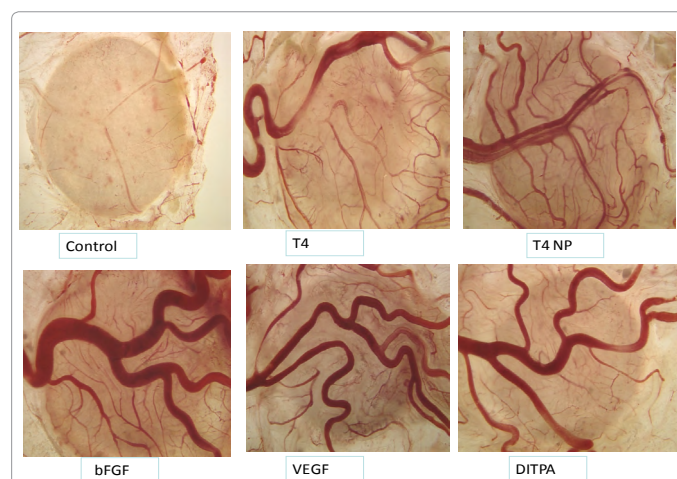


Figure 1: Photographic images of chick chorioallantoic membrane (CAM) assays of angiogenic activity of L-thyroxine (T_4), nanoparticulate T_4 (T_4 NP) [19], bFGF, VEGF and diiodothyropropionic acid (DITPA) [6], a thyroid hormone analogue. Control is phosphate-buffered saline. The methodology is described in Ref. 4. T_4 NP is a formulation in which the hormone is covalently-bound via a linker to a nanoparticle (poly[lactic-co-glycolic acid]); the nanoparticle precludes cellular uptake of the hormone and limits activity of T_4 to a receptor on the cell exterior (integrin $\alpha v\beta 3$) that regulates angiogenesis [13] and a variety of other cell functions [19]. The integrin is generously expressed by rapidly-dividing endothelial cells, as in this assay, and by tumor cells and osteoclasts [19]. Like T_4 and T_4 NP, DITPA initiates its proangiogenic activity at $\alpha v\beta 3$. Vascular branch points in the CAM assay may be quantified [4,6] to enable statistical comparisons of activities of biologic fluids, of different pharmacologic agents and of endogenous growth factors. Among the latter are bFGF and VEGF, shown here. We propose the use of the assay for measurement of pro-angiogenic activity in human blood that may oppose cancer chemotherapy that is primarily anti-angiogenic.

nonmalignant cells and platelets express the activated integrin only in small amounts [19].

Testing for the Contribution of Endogenous Thyroid Hormone to Cancer-relevant Angiogenesis

The CAM system not only indexes tumor-related angiogenesis, but permits spherical tumor growth of the implanted cells. Spherical growth is important to the development of a hypoxic cell population (JT Leith: unpublished observations) that resembles that of tumor behavior clinically and that supports radioresistance. Aggressive xenograft behavior in the CAM model includes metastasis to the chick embryo in the system [12]. These qualities of the assay are desirable in a cancer modeling system.

Vessel branch points are readily quantitated in the CAM system by analysis with standardized software of digitized images [4], permitting statistical evaluation of possible pro-angiogenic ('anti-angioinhibition') effects of patient sera in presence and absence of chemotherapeutic agent(s) [4,13]. The fertilized eggs are commercially available. The model can be exploited in multiple strategies, as discussed below.

In grafts of human lung cancer to the CAM exposed to tetrac, tumor vascularity is promptly reduced [7-9]. The CAM is euthyroid because of the contribution of maternal thyroid hormone to the egg and the maturation of the embryonic pituitary-thyroid axis by the time of application of tetrac to the model at 10-12 days of development *in ovo*. The results obtained with tetrac are consistent with loss of pro-angiogenic activity of thyroid hormone. But, as mentioned above, tetrac also may affect the angiogenic activities of VEGF [35] and bFGF [4,35], in the absence of T_4 and T_3 . The endpoint in studies of the pro-angiogenic action of thyroid hormone is histologic examination of the grafts for degree of vascularity.

It may also be desirable to study the behavior of established tumor grafts in a CAM rendered hypothyroid or to determine the interplay of pro-angiogenic properties of thyroid hormone and anti-angiogenic pharmaceuticals in the hypothyroid CAM system. We suggest that the 'euthyroid' CAM model containing an established human cancer implant may be rendered acutely hypothyroid as needed by exchange transfusion of embryonic serum treated with anion exchange resin [45]. Acute reductions in circulating endogenous thyroid hormone are required because established hypothyroidism will result in arrested tumor cell proliferation and decreased release of vascular growth factors; these factors will reduce detectability of anti-angiogenic activity of an added pharmaceutical or biological.

Specific strategies for use of the CAM in the context of anti-angiogenesis therapy include the following: (1) estimation of relapsed patient pro-angiogenic ('anti-angioinhibitory') activity in the absence of tumor grafts in the CAM, when first-line anti-angiogenic treatment has failed or when anti-angiogenesis is being considered as second-line therapy and specific treatment agent activity can be measured in the CAM in the presence of patient serum samples; (2) estimation of host pro-angiogenic activity in standard tumor cell line grafts in the CAM that are relevant to specific, relapsed patients—the assays also test specific treatment agents; (3) estimation of host pro-angiogenic activity in the presence of biopsied cell grafts from the patient in the CAM, with and without specific anti-angiogenic/chemo-therapeutic agent(s). Each of these approaches personalizes care.

In the clinical setting, several other paradigms can be considered for evaluating the potential contribution of endogenous (host) thyroid

hormone to the impact of therapeutic angioinhibition. These potential approaches include a search for correlations between circulating free T_4 levels and anti-tumor effectiveness of anti-angiogenic agents. In the absence of anti-angiogenic therapy, it may also be useful to attempt to quantitate the contribution of endogenous thyroid hormone to tumor vasculature. This strategy would involve estimation histologically of the ratio of tumor vascularity: tumor cellularity in biopsies from hypothyroid vs. euthyroid cancer patients at the same institution in protocols such as that for the breast cancer population characterized at MD Anderson Cancer Center by Cristofanilli and co-workers [22] and the glioblastoma patient cohort at the Cleveland Clinic in which biochemical hypothyroidism was medically induced [21]. Blood free T_4 levels at the times of biopsy should be included in the analysis.

Discussion

The pro-angiogenic properties of thyroid hormone have been satisfactorily demonstrated in several model systems [3,4,46-48]. That endogenous circulating thyroid hormone may clinically oppose the anti-angiogenic chemotherapy of cancer in patients has not been considered. We have proposed that the levels of hormone, as T_4 /free T_4 , may be sufficiently high in certain cancer patients to blunt the effect of therapy specifically directed at one or several vascular growth factors and thus limit the anti-tumor effectiveness of the therapeutic agent. We cite T_4 specifically here because (1) the NTI syndrome which cancer patients may exhibit is associated with low serum T_3 concentrations and (2) the effect of T_3 on tumor cell proliferation is less than that of thyroxine. We speculate that levels of T_4 in the upper quartile of the normal range or that are sufficient to depress circulating TSH ('subclinical hyperthyroidism') may be adequate to oppose pharmacologic angioinhibition by multiple mechanisms. Only prospective, controlled testing of medical induction of chemical hypothyroidism—raising the serum TSH to the upper limit of the reference range or above, but avoiding clinical symptoms of hypothyroidism—will satisfactorily address this issue. This has been done systematically in the setting of endstage glioblastoma multiforme, a highly vascular tumor, with reasonable success [21]. Coincident hypothyroidism appears to have changed the course of breast cancer in one large clinical experience [22], as noted above, reducing the aggressiveness of the disease. The latter study was not designed to segregate possible effects of decreased thyroid function on tumor cells or on tumor-associated vasculature, nor was the recent observation that incidental induction of hypothyroidism by tyrosine kinase inhibitors as a side effect is an important contributor to effect on another vascular tumor, renal cell carcinoma [24].

The receptor for thyroid hormone and hormone analogues on integrin $\alpha v \beta 3$ permits thyroid hormone to affect a number of regulatory factors in the process of angiogenesis, including function of multiple vascular growth factors [19], their receptors and angiogenic chemokines [49]. Small molecules such as bradykinin and angiotensin II are pro-angiogenic and their activity in this regard is modulated by the integrin (SA Mousa: unpublished observations). As noted above, the receptor on $\alpha v \beta 3$ influences endothelial cell motility (SA Mousa: unpublished observations) and vascular microtubule formation [5]. A prototypical inhibitor of thyroid hormone at the receptor on the integrin is Nanotetrac and this experimental nanoparticulate formulation of tetrac is effective as an anti-angiogenic substance in multiple model assays [19].

The second component of the pituitary-thyroid hormonal axis,

polypeptide thyrotropin (TSH) also supports vascular endothelial growth factor (VEGF) expression in thyroid cancer [50] and normal thyroid tissue [51]. Interestingly, a TSH receptor also exists on normal human dermal vascular endothelial cells; the receptor promotes VEGF expression in such cells in response to TSH [32]. Because primary hypothyroidism, accompanied by increases in serum TSH, appears to improve the clinical courses of certain important tumors—glioblastoma, breast, kidney, as mentioned above—it is unlikely that elevated circulating TSH is an important patient factor in reduced effectiveness of anti-angiogenic treatment of cancers.

In the current paper we propose the experimental use of the CAM assay in several paradigms to estimate anti-angiogenic properties of blood samples from cancer patients. The CAM model system also lends itself to screening of grafted tumors whose cells are harvested from individual patients for susceptibility to available anti-angiogenic clinical agents. Such studies would reflect the intrinsic susceptibility or resistance to angioinhibition of tumors established in the CAM from individual subjects, independent of circulating host factors such as thyroid hormone. But patient tumors established in the CAM system may be re-exposed to donor (host) serum or plasma in the model in the presence and absence of chemotherapeutic agents proposed for use in an example of personalization of tumor management.

In an extension of studies by the present authors of the effects of thyroid hormone analogues on radiosensitivity of tumor cells established in cell culture [52,53], Leith has demonstrated that assessment of tumor graft radiosensitivity is also feasible in the CAM model (JT Leith: unpublished observations).

The advantages of the CAM model include its history of use to estimate pro- and anti-angiogenic treatment agents in industry and academic centers, its standardization as a *quantitative* model of angiogenesis, its ready tolerance and support of human tumor cells and the low cost from commercial sources of 10 day-old chick embryos (fertilized eggs). The CAM summates actions of endogenous pro-angiogenic factors, including thyroid hormone, providing more information than individual assays for one or several possible anti-angiogenic factors. The system requires as many as 6-8 eggs per experimental variable, is thus not a high-throughput system, and of course has not been validated for the purposes proposed here.

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