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Proangiogenesis Action of the Thyroid Hormone Analog 3,5-Diiodothyropropionic Acid (DITPA) Is Initiated at the Cell Surface and Is Integrin Mediated

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We have recently described the proangiogenesis effects of thyroid hormone in the chick chorioallantoic membrane (CAM) model. Generation of new blood vessels from existing vessels was promoted 2- to 3-fold by either T_4 or T_3 at 10^{-8}–10^{-7} M total hormone concentrations. In the present studies, nanomolar concentrations of 3,5-diiodothyropropionic acid (DITPA), a thyroid hormone analog with inotropic but not chronotropic properties, exhibited potent proangiogenic activity that was comparable to that obtained with T_3 and T_4 in both the CAM model and in an *in vitro* three-dimensional human microvascular endothelial sprouting assay. The proangiogenesis effect of DITPA was inhibited by tetraiodothyroacetic acid, a thyroid hormone analog that competes with T_4 and T_3 for a novel cell surface hormone receptor site on integrin $\alpha v\beta 3$. The thyroid hormone analogs DITPA, T_4, and T_4-agarose, as well as basic fibroblast growth factor (b-FGF) and vascular endothelial cell growth factor, demonstrated comparable proangiogenic effects in the CAM model and in the three-dimensional human microvascular endothelial sprouting model. The proangiogenesis effect of either DITPA or b-FGF was blocked by PD 98059, an inhibitor of the ERK1/2 signal transduction cascade. Additionally, a specific integrin $\alpha v\beta 3$ small molecule antagonist, XT199, effectively inhibited the proangiogenesis effect of DITPA and b-FGF. Thus, the proangiogenesis actions of thyroid hormone and its analog DITPA are initiated at the plasma membrane, apparently at integrin $\alpha v\beta 3$, and are MAPK dependent. (*Endocrinology* 147: 1602–1607, 2006)

CONTROL OF ANGIOGENESIS is a complex process involving local release of vascular growth factors, the extracellular matrix, adhesion molecules, and metabolic factors (1–3). Mechanical forces within blood vessels may also play a role (2). The principal endogenous growth factors implicated in new blood vessel growth are the fibroblast growth factor (FGF) family and vascular endothelial growth factor (VEGF) (4). The MAPK/ERK1/2 signal transduction cascade is known to be involved both in VEGF gene expression and in control of proliferation of vascular endothelial cells (4).

The evidence for a proangiogenic effect of systemically administered thyroid hormone was initially based upon histologic and physiologic evidence developed in animal models (5); the possible molecular mechanisms of this action of the hormone, however, were not defined. We have recently shown that thyroid hormone (T_4) enhances the activation of MAPK by basic FGF (b-FGF) in endothelial cells, and, further, the hormone stimulates the expression of b-FGF by endothelial cells (3). These effects together contribute in large measure to a significant stimulatory effect of T_4 on angiogenesis in the chorioallantoic membrane (CAM) assay (3).

We have shown that the thyroid hormones T_3 and T_4 are both proangiogenic (3) and further, that T_4-agarose, a formulation of the hormone limited to activity at the plasma membrane, is also a potent stimulus for angiogenesis (3). The thyroid hormone analog, tetraiodothyroacetic acid (tetrac), which is known to inhibit thyroid hormone binding to plasma membranes, blocks T_4-induced angiogenesis in the CAM model, but is not itself proangiogenic (3). A plasma membrane receptor for T_4 has recently been identified on integrin $\alpha v\beta 3$ (6). We have found that T_3 and tetrac both displace T_4 from purified integrin $\alpha v\beta 3$ (6); these results are consistent with findings in the CAM studies suggesting that the hormones and analog are interacting with the same receptor (3).

Coordinated angiogenesis and cardiac growth have been described by several authors in response to T_4-induced myocardial hypertrophy in the rat (5, 7). The appearance of increased capillary numerical density preceded hypertrophy in the latter model (7). Diiodothyropropionic acid (DITPA) is a thyroid hormone analog developed for use in the treatment of heart failure (8). The same group also showed that DITPA promoted angiogenesis in a rat model of cardiac hypertrophy previously subjected to experimental myocardial infarction (9).

The availability of a chick CAM model of angiogenesis (3, 10) and a three-dimensional (3-D) human microvascular endothelial cell sprouting assay provided us with two systems in which to quantitate angiogenesis and to study mecha-

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Abbreviations: b-FGF, Basic FGF; CAM, chorioallantoic membrane; 3-D, three-dimensional; DITPA, 3,5-diiodothyropropionic acid; EBM, endothelial basal medium; EC, endothelial cell; FGF, fibroblast growth factor; HDMEC, human dermal microvascular endothelial cells; tetrac, tetraiodothyroacetic acid; TR, thyroid hormone receptor; VEGF, vascular endothelial growth factor.

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nisms involved in induction of angiogenesis by thyroid hormone and the hormone analog, DITPA. In this report, we describe a proangiogenesis effect of DITPA that approximates that of b-FGF in the CAM model. We also provide evidence that the proangiogenic effect of DITPA is initiated at the endothelial cell plasma membrane, involves a plasma membrane integrin $\alpha v\beta 3$ receptor, and is mediated by activation of the ERK1/2 signal transduction pathway.

Materials and Methods

Reagents

T_4, T_3, tetrac, T_4-agarose, and DITPA were obtained from Sigma Chemical Co. (St. Louis, MO) and PD 98059 from Calbiochem (La Jolla, CA). Polyclonal anti-b-FGF was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant b-FGF and VEGF were obtained from Invitrogen (Carlsbad, CA), and purified human fibrinogen from the New York Blood Bank (New York, NY). The high-affinity small molecule $\alpha v\beta 3$ antagonist, XT199, is available at the Pharmaceutical Research Institute (Albany, NY).

In vitro 3-D sprouting assay of angiogenesis using human dermal microvascular endothelial cells cultured on microcarrier beads coated with fibrin

A microcarrier *in vitro* angiogenesis assay previously developed to investigate bovine pulmonary artery endothelial cell (EC) angiogenesis behavior in bovine fibrin gels (11, 12) was modified for the study of human microvascular EC angiogenesis in 3-D extracellular matrix environments. The protocol for this assay is illustrated in Fig. 1. Confluent human dermal microvascular endothelial cells (HDMEC; passages 5–10) were mixed with gelatin-coated Cytodex-3 beads at a ratio of 40 cells per bead. Cells and beads (150–200 beads/well for each 24-well plate) were suspended in 5 ml endothelial basal medium (EBM) plus 15% normal human serum and mixed gently every hour for the first 4 h; the mixture was then cultured overnight in a CO_2 incubator. The next day, 10 ml of fresh EBM with 15% human serum were added for another 3 h. Before each experiment, 500 μl of PBS and 100 μl of the EC-bead culture solution were added to each well of a 24-well plate. The number of beads/well was determined, and the concentration of beads/EC was calculated.

Human fibrinogen, isolated as previously described (13) and available at the New York Blood Bank (Stony Brook, NY), was dissolved in EBM and sterilized through a 0.22-μm filter. A solution of pure fibrinogen (1 mg/ml) (pH 7.4) was prepared, with or without the agents to be tested. For a positive control, a mixture of standard growth factors (30 ng/ml VEGF + 25 ng/ml b-FGF) was used. EC-beads were washed twice with EBM, and then added to the fibrinogen solution. For each condition, the experiment was carried out in triplicate. EC beads in fibrinogen solution were mixed gently, and 2.5 μl human thrombin (0.05 U/μl) were added. Immediately, a 300-μl aliquot from each sample was transferred to a well of the 24-well plate. Polymerization of the fibrinogen solution occurred in 5–10 min; after 20 min, EBM with 20% normal human serum and 10 μg/ml aprotinin were added. The plate was incubated in a CO_2 incubator for 24–48 h or for a longer time to allow for the HDMEC to invade the fibrin gel and form tubes.

The angiogenesis response was monitored visually and recorded by video image capture. Specifically, capillary sprout formation was observed and recorded with a Nikon Diaphot-TMD inverted microscope (Nikon, Inc., Melville, NY) equipped with an incubator housing with a Nikon NP-2 thermostat and Sheldon (Cornelius, OR) no. 2004 carbon dioxide flow mixer. The microscope was directly interfaced to a video system consisting of a Dage-MTI (Michigan City, IN) charge-coupled device-72S video camera and a Sony (New York, NY) 12" PVM-122 video monitor linked to a computer. The images were captured at various magnifications using Adobe Photoshop (San Jose, CA). The effect of the proangiogenesis factors on sprout angiogenesis was quantified visually by determining the number and percent of EC-beads with capillary sprouts. One hundred beads (five to six random low-power fields) in each of triplicate wells were counted for each experimental condition. All experiments were repeated at least three times. To locate the nucleus of HDMEC, the fibrin or collagen gels were fixed by methanol/acetone (1:1) and stained with 0.001% phalloidin iodine, a fluorescence-labeled actin that binds to the nucleus.

CAM model of angiogenesis

Neovascularization was examined in the CAM model, as previously described (3, 10, 14–17). Ten-day-old chick embryos were purchased from Spafas, Inc. (Preston, CT) and incubated at 37 C with 55% relative humidity. With a hypodermic needle a small hole was made in the shell at the air sac, and a second hole was made on the long side of the egg, directly over an avascular portion of the embryonic membrane identified by candling. A false air sac was created beneath the second hole by distal application of negative pressure, so that the CAM separated from the shell. A window approximately 1.0 cm^2 was cut in the shell over the dropped CAM, allowing direct access to the underlying membrane.

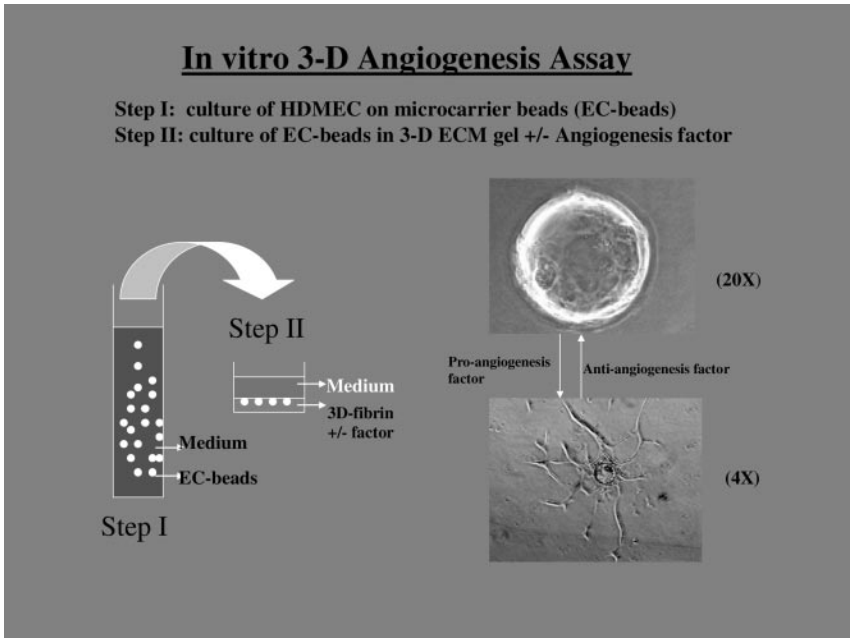


FIG. 1. Illustration of the 3-D *in vitro* sprouting assay procedure for human microvascular endothelial cell angiogenesis on fibrin-coated beads. Details of the procedure are described in *Materials and Methods*.

b-FGF (1 μg/ml) was used as a standard proangiogenic agent. Sterile disks of no. 1 filter paper (Whatman International, Kent, UK) were pretreated with 3 mg/ml cortisone acetate and air dried under sterile conditions. Thyroid hormone and analogs (T_4, agarose-T_4, DITPA), b-FGF, VEGF or control vehicle, and/or inhibitors were then applied to the disks, and the disks allowed to dry, then suspended in PBS and placed on the CAMs. Filters treated with thyroid hormone and analogs, DITPA, b-FGF, or VEGF, alone or in combination, were placed on the first day of the 3-d incubation, and antibody to b-FGF added 30 min later to selected samples. At 24 h, either the MAPK cascade inhibitor PD 98059, tetrac, or the $\alpha v\beta 3$ integrin antagonist XT199 was also added to the CAMs topically, by means of the filter disks. None of these agents, applied alone, affected angiogenesis.

Microscopic analysis of CAM sections

After incubation at 37 C with 55% relative humidity for 3 d, the CAM tissue directly beneath each filter disk was resected from each CAM sample. Tissues were washed three times with PBS, placed in 35-mm Petri dishes (Nalge Nunc, Rochester, NY), and examined under an SV6 stereomicroscope (Carl Zeiss, Thornwood, NY) at ×50 magnification. Digital images of CAM sections exposed to the treatment filters were collected using a three-charge-coupled device color video camera system (Toshiba America, New York, NY), and analyzed with Image-Pro software (Media Cybernetics, Silver Spring, MD). The number of vessel branch points contained in a circular region equal to the area of each filter disk was counted. One image was counted in each CAM preparation, and findings from eight CAM preparations were analyzed for each treatment condition. In addition, each experiment was carried out three times. The resulting angiogenesis index is the mean ± SD of new branch points in the collected samples from each treatment condition.

Statistical analysis

Statistical analysis was performed by one-way ANOVA using Statview software (Adept Scientific, Acton, MA), comparing the mean ± SD of branch points from each experimental group with its respective control group. Statistical significance was defined as $P < 0.05$. In the CAM studies, the angiogenesis index for each treatment group was compared with the corresponding control group. The effects of DITPA, b-FGF, VEGF, T_4, and T_4-agarose were compared with samples treated with PBS, alone, or with the appropriate control group, *e.g.* DITPA with and without inhibitor. The effect of each inhibitor on DITPA-induced angiogenesis was calculated by determining the percent reduction in the DITPA effect (mean ± SD) caused by each inhibitor, compared with the angiogenesis seen with DITPA alone, using the following formula:

$$\text{Inhibition of angiogenesis (\% inhibition} \pm \text{SD)} = \left\{1 - \frac{[(\text{DITPA} + \text{inhibitor}) - \text{PBS}]}{(\text{DITPA} - \text{PBS})}\right\} \times 100$$

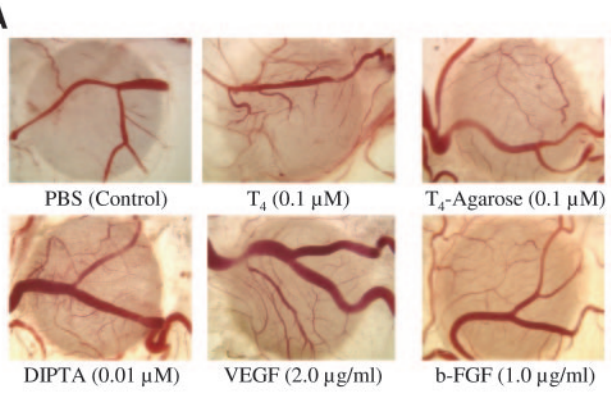
Results

Effect of thyroid hormone analogs on angiogenesis

DITPA, T_4, and T_4-agarose each caused stimulation of angiogenesis in the CAM model that was comparable to that obtained with b-FGF or VEGF (Fig. 2A). The results of three similar experiments are summarized in Fig. 2B. The proangiogenic effects of T_4, T_4-agarose, and DITPA were maximal and comparable at a concentration of 0.1 μM for each analog. DITPA at 0.01 μM stimulated angiogenesis to a lesser degree, although still significant. Results with T_4 and DITPA were similar in magnitude to those obtained with b-FGF (1.0 μg/ml) and VEGF (2.0 μg/ml).

Effect of tetrac on DITPA-induced angiogenesis

It has been previously shown that T_4 promotes angiogenesis by a plasma membrane-initiated mechanism and that this proangiogenic action is blocked by a deaminated iodothyronine analog, tetrac, which is known to inhibit binding of T_4 to plasma membranes (3). In the CAM model, the addition of tetrac (0.1 μM) inhibited the action of DITPA (Fig. 3). Tetrac, alone, was not proangiogenic but has been shown to block the proangiogenic action of T_4 (3).



B

Treatment	Mean Vessel Branch Points ± SD
PBS (Control)	87 ± 9
T_4 (0.1 μM)	148 ± 7*
T_4-agarose (0.1 μM)	167 ± 8*
DITPA (0.01 μM)	134 ± 11*
DITPA (0.1 μM)	170 ± 9*
VEGF (2.0 μg/ml)	168 ± 10*
b-FGF (1.0 μg/ml)	174 ± 8*

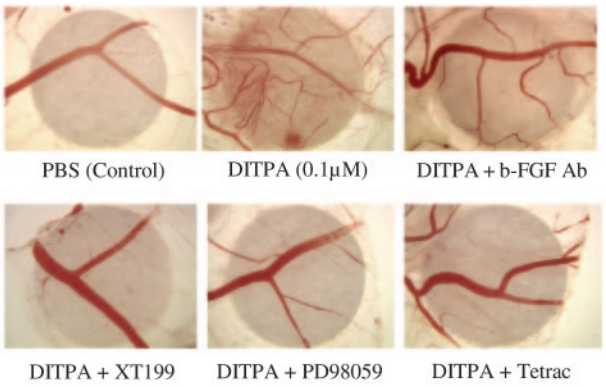
Data represent mean ± SD, n=8; *p<0.01, indicating significant stimulation of angiogenesis. PBS, phosphate-buffered saline.

FIG. 2. Stimulation of angiogenesis in the chick CAM model by DITPA, T_4, and T_4-agarose in comparison to b-FGF and VEGF. Membranes were treated for 3 d as described in *Materials and Methods*. A, Results from a representative experiment are shown (n = 8–10 images/group/experiment in three different experiments). Stimulation of angiogenesis by T_4, T_4-agarose, DITPA, b-FGF and VEGF is evident. B, The results of three experiments are summarized in the *table* as the angiogenesis index (mean ± SD of branch points per field) for each experimental variable.

b-FGF is a mediator of DITPA-induced angiogenesis

We have previously demonstrated that thyroid hormone stimulation of angiogenesis is mediated by enhancement of b-FGF expression in endothelial cells, and that the stimulation of angiogenesis by T_4 or b-FGF is blocked by the simultaneous addition of b-FGF antibody to the CAM (3). To define the role of b-FGF more precisely in stimulation of angiogenesis by DITPA, a polyclonal antibody to b-FGF was added to filters treated with DITPA, and angiogenesis was measured after 72 h. In results shown in Fig. 3, the b-FGF antibody inhibited angiogenesis stimulated by DITPA in the absence of exogenous b-FGF, suggesting that the proangiogenesis effect of this thyroid hormone analog in the CAM model was largely mediated by increased b-FGF expression by the CAM

A



B

Treatment	Mean Vessel Branch Points ± SD	Mean % Inhibition ± SD
PBS (Control)	95.0 ± 9.7	-
b-FGF (1.0 μg/ml)	154.5 ± 12.2	-
DITPA (0.1 mM)	169.8 ± 9.5	-
DITPA (0.1 mM) + anti-b-FGF	104.1 ± 6.8	86.3 ± 7.4*
DITPA (0.1 mM) + XT199	110.0 ± 4.0	79.9 ± 5.3*
DITPA (0.1 mM) + PD98059	103.8 ± 8.7	88.2 ± 11.6*
DITPA (0.1 mM) + Tetrac	123.7 ± 8.7	61.7 ± 11.7*

Data are mean ± SD, n = 8; *p <0.01, indicating significant inhibition of the DITPA effect.

FIG. 3. Effect of tetrac, PD98059, XT199, and b-FGF antibody on DITPA stimulation of angiogenesis in the chick CAM model. A, Membranes were treated for 3 d as described, and a representative image from three studies including each variable is shown. A 2-fold increase in blood vessel branch formation is seen in a representative image from preparations exposed to 0.1 μM DITPA for 3 d, an effect that is inhibited by tetrac (0.075 μg/ml or 0.1 μM). Tetrac inhibition of the DITPA effect is similar to that shown previously with inhibition of the effects of T_4 or T_4-agarose on angiogenesis (3). Tetrac, alone, does not affect angiogenesis. The inhibitor PD 98059 (5.3 μg/ml), a MAPK (ERK1/2) signal transduction cascade inhibitor, also blocked the effect of DITPA, as did the integrin αvβ3 inhibitor, XT199 (4.8 μg/ml), and the antibody to b-FGF (10 μg/ml). The latter effect indicates the ultimate role of b-FGF in the stimulation of angiogenesis by DITPA and is similar to findings with T_4 (3). B, The pooled results of three experiments are summarized in the *table* as the angiogenesis index (mean ± SD of branch points per field) for each experimental variable.

endothelial cells (3). The b-FGF antibody applied alone had no effect on angiogenesis in this model (results not shown).

The ERK1/2 signal transduction pathway in stimulation of angiogenesis by DITPA

Studies of ERK1/2 inhibition were also carried out in the CAM assay, and representative results are shown in Fig. 3. DITPA (0.1 μM) caused a 2-fold increase in blood vessel branching, a response that was effectively blocked by the ERK1/2 activation inhibitor, PD 98059. We have previously shown that b-FGF stimulation of branch formation was also blocked by this inhibitor of ERK1/2 activation (3). PD 98059 applied alone did not affect angiogenesis (results not shown).

The role of integrin αvβ3 in DITPA-mediated angiogenesis

As indicated above, the plasma membrane receptor for thyroid hormone is integrin αvβ3 (6). The integrin αvβ3 antagonist XT199 is known to inhibit T_4 stimulation of angiogenesis in the CAM assay, but does not itself affect basal angiogenesis (17). In a representative study illustrated in Fig. 3, stimulation of angiogenesis caused by DITPA was totally blocked by XT199. In additional studies, the αvβ3 monoclonal antibody LM609 also blocked DITPA-induced angiogenesis (data not shown). Thus, the proangiogenic effect of DITPA is initiated at the plasma membrane integrin αvβ3 and involves activation of the ERK1/2 pathway to promote b-FGF release from endothelial cells in a manner similar to the effect of T_4. ERK1/2 activation is secondarily required to transduce the b-FGF signal and cause new blood vessel formation.

Studies with DITPA in a 3-D sprouting assay

DITPA promoted angiogenesis 2.1-fold in the 3-D microvascular endothelial cell sprouting assay (Table 1). A 2.1-fold stimulation in the mean number of migrated cells and a 2.0-fold increase in mean microvessel length were seen. These significant results were similar to those seen after treatment with a combination of b-FGF and VEGF (2.4- and 2.6-fold stimulation in cell number and microvessel length, respectively). These effects of DITPA were all reduced by tetrac, by the αvβ3 antagonist XT199, and by the ERK1/2 activation inhibitor, PD98059 (Table 1). These results indicate again that DITPA acts at the cell membrane receptor, integrin αvβ3, promoting activation of a MAPK-dependent pathway and stimulation of angiogenesis.

Discussion

Enhancement by the thyroid hormone analog DITPA of the angiogenesis-promoting activity of b-FGF and VEGF in the CAM and 3-D human endothelial cell sprouting assays was demonstrated in the present experiments. DITPA at 10^{-7} M

TABLE 1. Inhibition of the proangiogenic effect of DITPA by either a MAPK pathway inhibitor, tetrac or an integrin αvβ3 antagonist, shown in the 3-D human microvascular endothelial sprouting assay

Treatment	Mean number of migrated cells ± SD	Mean length (mm) ± SD
Control	88 ± 14	0.47 ± 0.06
b-FGF (30 ng/ml) + VEGF (25 ng/ml)	213 ± 16[a]	1.25 ± 0.12[a]
DITPA (0.1 μM)	186 ± 16[a]	0.96 ± 0.07[a]
DITPA (0.1 μM) + tetrac (0.15 μg/ml)	107 ± 10	0.65 ± 0.05
DITPA (0.1 μM) + PD98059 (3 μg/ml)	90 ± 8	0.49 ± 0.04
DITPA (0.1 μM) + XT199 (2 μg/ml)	87 ± 8	0.44 ± 0.04

HDMVCs were pretreated with b-FGF and VEGF, with DITPA alone, or with DITPA in the presence of tetrac, PD98059, or XT199 in the concentrations indicated. Images were taken at ×4 and ×10 on d 3, and the mean number of migrated cells and mean blood vessel length determined. Shown are the results of three similar experiments, comparing findings from studies with the growth factor combination (positive control) with results of studies with DITPA, with or without inhibitors of DITPA action.

[a] $P < 0.01$, indicating significant increase from control either in cell migration, or in blood vessel length.

was comparable in angiogenic effect to the concentrations of VEGF and b-FGF that are maximally effective in these models. New blood vessel growth in the rat heart has been reported to occur concomitantly with induction of myocardial hypertrophy by systemic administration of T_4 (7, 18). However, the hormone dose in such studies was considerably higher than replacement T_4 in man, and thyroid hormone has not been widely regarded to be an angiogenic factor (9). Zheng *et al.* (19) have reported that DITPA, a thyroid hormone analog developed as an inotropic agent, is angiogenic in a postinfarction rat heart model. The present report, based on both chicken and human endothelial cell models, establishes that DITPA, at a concentration comparable to circulating total T_4 levels in humans, is proangiogenic in settings other than the heart. Additionally, Zheng *et al.* (19) and others (20, 21) have shown that local increases in protein concentrations of b-FGF, VEGF, angiopoietin, and Tunica interna endothelial cell kinase (TIE-2) occur with high-dose DITPA administration. Here we show that release of b-FGF from DITPA-treated endothelial cells is required for the proangiogenesis action of a relatively low concentration of DITPA. In the models tested above, anti-b-FGF completely blocked the stimulatory action of DITPA in these studies. This antibody is also known to block the proangiogenic effect of T_4 (3).

A critical feature of the present observations, however, is that the angiogenic activity of DITPA in the two assays used is initiated at a novel cell surface receptor site for thyroid hormone that we have recently described on integrin $\alpha v\beta 3$ (6). It is not surprising that the complex proangiogenesis response of DITPA is plasma membrane initiated because we have shown that the angiogenic response to T_4 also appears to begin at the integrin cell surface receptor site (6). DITPA and T_4 have similar dose-response relationships in the CAM model. Whereas DITPA is known to bind to the nuclear thyroid hormone receptor (TR) and induces expression of a cDNA microarray similar to, but not identical with, that of T_3 (22), the analog binds to TR with relatively low affinity (22). This also suggests that the angiogenic activity of DITPA does not require a primary interaction of the analog with TR. We have shown elsewhere that angiogenesis can be induced by agarose-T_4, a formulation of the hormone that does not gain access to the cell interior (3, 17) and thus does not interact with TR in the intact cell.

Because ambient concentrations of thyroid hormone in the intact organism are relatively constant, it is possible that the hormone may be a permissive factor for the growth of new blood vessels. The proangiogenic effect of thyroid hormone may be desirable for stimulation of angiogenesis in the heart in the presence of arterial narrowing, in peripheral vascular disorders, or in the clinical context of wound healing. DITPA may be an attractive therapeutic thyroid hormone analog for this purpose. In contrast, in proliferative neovascularization in the eye (*e.g.* diabetic retinopathy), a proangiogenic contribution of thyroid hormone analogs would be undesirable. Angiogenesis associated with primary or metastatic tumors might also be stimulated by iodothyronines. These complications, however, are potentially subject to inhibition by tetrac or by an antiintegrin small molecule, such as XT199. Cellular models are available in which to test these possible consequences of thyroid hormone action. The possibility of systemic, noncardiac proangiogenic effects of DITPA may be considered in situations where there is an indication for stimulation of neovascularization in the presence of existing heart failure (23).

The desirability of local short-term delivery of DITPA or other proangiogenic thyroid hormone analogs within the coronary circulation, *e.g.* via hormone-coated stents, is apparent. In the case of DITPA, an angiogenic response may support the attractive inotropic actions of the analog that interestingly occur without increasing heart rate (23, 24).

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