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The Stimulative Effect of T3 and T4 on Human Myocardial Endothelial Cell Proliferation, Migration and Angiogenesis

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Abstract

Angiogenesis, the process involving the growth of new blood vessels from pre-existing vessels, may be a target for combating diseases characterized by either poor vascularisation or abnormal vasculature as in cardiovascular diseases. Thyroid hormones (THs) are strong proangiogenic factors whose action starts at the plasma membrane integrin αvβ3 protein transducing rapid nongenomic signals on tumor thyroid cells. The tetraiodothyroacetic acid (Tetrac) inhibits the binding of TH to integrin receptor αVß3 blocking angiogenesis. Growing evidences suggest that, also in heart, Triiodothyronine (T3) and Thyroxine (T4) triggered nongenomic pathways through their binding to integrin receptor αVβ3 inducing capillary proliferation. The angiogenic activity of T3 and T4 has been studied by the chick choriallontoic endothelial cell microtubule assay and the human dermal microvascular endothelial cells microtubule assay.

The aim of this work was to evaluate the direct stimulative activity of T3 and T4 on human cardiac microvascular endothelial cell (HMVEC-C) proliferation, migration and tube formation, employing Tetrac as inhibitor. Our *in vitro* study indicates that T3 and T4 directly stimulate angiogenesis in HMVEC-C observed as capillary density, cell proliferation and cell migration. In all models, Tetrac (5 μM) inhibited the proangiogenic effect of T3 and T4 suggesting its integrin-mediated action. Sq RT-PCR assay revealed that T3, and in less extent T4, increased the expression of angiogenic genes such as angiopoietin-1 (Angpt-1), angio-associated migratory cell protein (AAMP) and vascular endothelial growth factor (VEGF), whereas when the cells were pre-incubated with Tetrac this effect was abolished. In conclusion, our results show that THs stimulate angiogenesis, suggesting a potential therapeutic role aimed at increasing capillary density in cardiac diseases.

Keywords: Thyroid hormones; Human microvascular endothelial cardiac cell; Angiogenesis

Abbreviations: THs: Thyroid Hormones; Tetrac: Tetraiodothyroacetic Acid; T3: Triiodothyronine; T4: Thyroxine; HMVEC-C: Human Cardiac Mycrovascular Endothelial Cell; DITPA: Diiodothyropropionic Acid; Angpt-1: Angiopoietin-1; AAMP: Angio-Associated Migratory Cell Protein; VEGF: Vascular Endothelial Growth Factor; hEGF: Human Endothelial Growth Factor; R3IGF1: Recombinant Long R Insulin Like Growth Factor; GA-1000: Gentamicin-Amphothericin; Sq RT-PCR: Semiquantitative Reverse Transcription; GAPDH: Glyceraldehyde 3-Phosphate; SDS-PAGE: Dehydrogenase Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; Angpt-2: Angiopoietin-2

Introduction

Angiogenesis is considered a physiological process involving the growth of new blood vessels from pre-existing vessels. It is also a fundamental step in the transition of tumors from a benign to a malignant condition, therefore, anti-angiogenic therapies are employed to fight cancer and malignancies [1,2]. At the same time, angiogenesis may be a target to treat diseases characterized by poor or abnormal vasculature, such as atherosclerotic diseases, wound healing disorders and myocardial infarction [3,4]. Thus, pro-angiogenic therapies have been explored as important options to treat cardiovascular diseases [5,6].

Thyroid hormones (THs) are strong proangiogenic factors whose action starts at the plasma membrane integrin αvβ3 protein [7-9], that belongs to a family of heterodimer receptors very important for interaction with extracellular matrix proteins [10-12]. In particular, it has been reported that the plasma membrane integrin receptor αVß3 is widely expressed on tumor thyroid cells trasducing rapid nongenomic TH signals into angiogenic response and cell proliferation through TH binding to its RGD site (Arg-Gly-Asp) [13,14]. Moreover, the thyroxine (T4)-derived metabolite, Tetrac, normally considered to be inactive on angiogenesis, has been reported to inhibit the binding of TH to integrin receptor αVß3 on RGD site in non-small lung cancer cells [15].

In addition to the classic genomic actions, there is growing evidence that in the heart T3 and T4 trigger nongenomic pathways through their binding to plasma membrane receptor leading to a rapid regulation of cardiac functions [16,17]. In experimental myocardial infarction, some studies indicate that TH treatment induce capillary proliferation after a few hours [18]. Other authors showed that diiodothyropropionic acid (DITPA), a thyroid hormone analogue induced blood vessel formation by VEGF and bFGF [19]. Moreover in the cardiomyopathic hamster, Gerdes et al. showed an increased myocardial blood flow and presumed an increased angiogenesis after T4 or DITPA treatment [20]. Recently, Chen J et al. showed that three day T3 treatment significantly induced cardiac capillary growth in hypothyroid mice and that T3 stimulation induced sprouting *in vitro* [21].

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So far, data on the angiogenic effect of T3 and T4 has been reported in two models: the chick choriallontoic membrane [13] and the human dermal microvascular endothelial cells [22,23]. Nevertheless, the molecular bases of the proangiogenic activity of THs are not yet fully clarified.

The aim of the present work was to evaluate the direct stimulative activity of T3 and T4 on the human cardiac mycrovascular endothelial cell (HMVEC-C) proliferation, migration and tube formation. As inhibitor of proangiogenic activity of TH we emplojed a T4 derivative, tetraiodothyroacetic acid (Tetrac), that has been shown to block thyroid hormone binding at the integrin receptor [12].

Materials and Methods

Reagents

T3, T4, Tetrac and VEGF were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Matrigel was purchased from BD Bioscience (San Jose, CA).

Cell cultures

Human microvascular endothelial cardiac cells (HMVEC-C, Lonza, Milan, Italy) were grown in basal medium (EBM; Lonza) supplemented with 5% fetal bovine serum, 0.1% human endothelial growth factor (hEGF), 0.04 hydrocortisone, 0.1% GA-1000 (gentamicin-amphothericin B), 0.1% vascular endothelial growth factor, 0.4% human fibroblast growth factor, 0.1% R3IGF1 (Recombinant long R insulin like growth factor) and 0.1% ascorbic acid. Cells at passage 5 were used for all experiments. All cell types were cultured at 37°C in 5% CO2 and media were replaced every two days.

Proliferation assay

Effect of T3 and T4 on HMVEC-C proliferation: Proliferation of cells was assayed by Alamar blue (Invitrogen Srl, Milano, Italy). Cells were seeded at a density of 3×10^3 in 96-well microplates coated with gelatin (2 mg/ml) and cultured in serum-free medium (EBM2 for HMVEC-C) at 48 hrs in the presence or absence of T3 (3 nM, 60 nM, 1 μM) or T4 (0.1 μM), preincubated or not with Tetrac for 15 min (5 μM). At the end of the incubation, 10 μl of Alamar blu were added to each well and the incubation continued for 4 h at 37°C. VEGF (1 μM and 4 μM) was used as positive control. Absorbances were measured by TECAN spectrophotometer (Infinite M200PRO; Switzerland) using a 570 nm wavelength.

RNA extraction and Sq RT-PCR

Cells were cultured in serum-free EBS in the presence or absence of T3 (1 μM) or T4 (0.1 μM) with or without Tetrac (5 μM) for 24 h at 37°C. Total RNA was isolated from pellets of about 2×10^5 cells using the TaqMan Gene Expression Cells-to-CT TM Kit (Applied Biosystems). Five μL of the cells suspensions were added to lysis buffer as described by the manufacturer. Two μL of the reverse transcribed samples (100 ng mRNA) were used for semiquantitative reverse transcription (Sq RT-PCR) amplification performed with GoTaq Green Mastermix (Promega, USA). The following primers were used: angiopoietin-1 (Angpt-1): forward-cagtggctgcaaaaacttga, reverse-tccacatctgtgagctttcg; angiopoietin-2 (Angpt-2): forward-gatcttgtcttggcctcagc, reverse-acggcgttagacatgtaggg; VEGF: forward-ctacctccaccatgccaagt, reverse-tttcttgcgctttcgttttt; angio-associated migratory cell protein (AAMP): forward-ctttgcattgcactcagcat, reverse-cagtcaccattcgggacttt; integrin ß3 forward-tgacatcgagctggtgaaag, reverse-gagtagcaaggccaatgagc; Glyceraldehyde 3-Phosphate (GAPDH): forward-agccactgctgtgcttttaag; reverse-ccaaaaccaatgatctcatcc. PCR products were visualized following electrophoresis in 1% agarose gels. Fragments of RT-PCR cDNA were purified from gels and sequenced by BMR-Genomics (Italy). Relative levels of cDNA genes were normalized to GAPDH.

Wound-healing assay

Migration of HMVEC-C cells was assayed by a scratched-wound assay. When the cells were become confluent in 24 well plates, a wound was made in the center of the cell monolayers with a tip. After two washes to remove debris, cells were incubated in the presence or absence of T3 (1 μM) or T4 (0.1 μM) with or without Tetrac (5 μM). VEGF (1 μM) was used as positive control. After 2 days, cultures were observed by microscopy (40 Carl Zeiss vision GMBH) and images captured using Axiovision 3.0.

Capillary and network formation with HMVEC-C cells

HMVEC-1 cells (5×10^4 cells/well) were seeded on Matrigel in 48-well cell plates and cultured in serum-free EBS in the presence or absence of T3 (1 μM) or T4 (0.1 μM) with or without Tetrac (5 μM). After 24 h, seven microscopic fields were photographed. VEGF (1 μM) was used as positive control. The number of capillary networks was visually counted in a blinded mode.

Western blotting

HMVEC-C cells were incubated with or without the addition of T3 (1 μM) for 24 h at 37°C. Cell lysates were prepared for western blot analysis as previously described [14]. Proteins (20-30 μg) were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically blotted onto 0.2 mm nitrocellulose membranes. The membrane were blocked for 1 h at room temperature with 1% blocking solution (Roche, IN, USA) in Tween-20/PBS 0.1% vol/vol (PBST) and then incubated overnight at 4°C with primary antibodies against integrin β3 (rabbit sc-4702 Santa Cruz, 1:1000) and GAPDH (mouse, Cell Signaling Technology, Danvers, MA, USA, 1:2000). After three washes, the appropriate secondary IgG-HRP linked conjugate (anti–rabbit A0545 Sigma; anti-mouse sc-2005 Santa Cruz) at 1:3000 dilutions was applied. Proteins were visualized with a chemiluminescence assay (Roche kit, IN, USA) and analyzed with Scion image software (Scion Corporation USA).

Statistical analysis

Differences between the means of two variables were evaluated by the Student's t test. Comparisons among more than two groups were evaluated by ANOVA. The results are expressed as mean ± SD and P value was considered significant when <0.05.

Results

Presence of integrin β3 receptor on HMVEC-C cells

The presence of integrin β3 was evaluated by Western blotting analysis and sq RT PCR on HMVEC-C. A clear band of approximately 110 kDa corresponding to the molecular mass of integrin β3 protein was detected; by PCR the corresponding gene expression was also observed. Treatment with T3 or T4 in presence or absence of Tetrac did not change the integrin β3 expression compared to cells in basal condition (Figure 1).

Proliferation effects of T3 and T4 on HMVEC-C cells

To evaluate whether T3 and T4 induced proliferation of

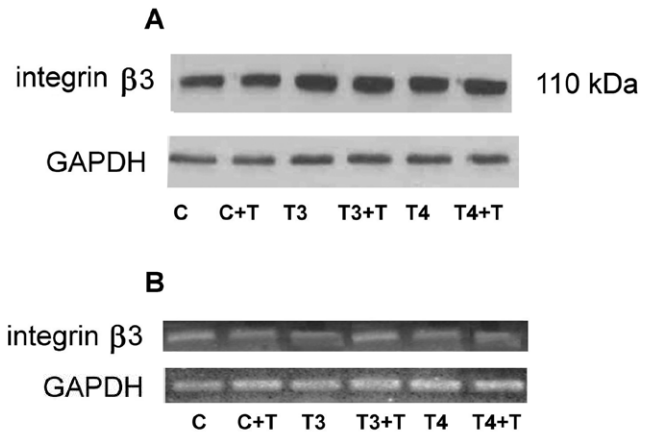

Figure 1: Presence of Integrin β3 receptor on HMVEC-C cells. Expression levels of Integrin β3 were determined by Western blot analysis (A) and by sq RT-PCR (B) in cells in the absence or presence of T3 (1 μM), T4 (0.1 μM) or Tetrac (T) (1 μM) after 24 hrs.

HMVEC-C, cells were treated in serum deprivation, with T3 (3 nM, 60 nM, 1 μM) or T4 (0.1 μM) for 48 h. Treatment with 60 nM and 1 μM T3 increased cellular proliferation when compared to untreated cells by 16% ($p<0.05$) and 45% ($p<0.01$), respectively. No significant increment was observed in presence of T3 (3 nM) that corresponds to a physiological concentration. Treatment with 0.1 μM T4 increased cellular proliferation by 83% ($p<0.01$) and treatment with 1 μM and 4 μM VEGF increased cellular proliferation by 26% ($p<0.01$), and 70% ($p<0.01$), respectively (Figure 2, open histograms). When the cells were preincubated with Tetrac (5 μM) the proliferative effect was abolished both for T3 (60 nM, 1 μM, $p<0.05$), T4 ($p<0.01$) and VEGF (1 μM), whereas was reduced by 34% for VEGF 4 μM (see Figure 2, gray histograms).

TH promotes capillary network formation in HMVEC-C cells

To examine the effects of TH on angiogenesis, we measured the capillary network formation of HMVEC-C cells seeded on Matrigel. Treatment with T3 (1 μM) caused a significant enhancement of the capillary network formation of 1.45 fold (45%) over basal condition (*$p<0.01$) and by T4 of 1.37 fold (37%) ($p<0.01$). Tetrac abolished the increment of capillary density induced by T3 ($p<0.01$) and by T4 ($p<0.01$). VEGF (1 μM) increased capillary density of 42% that was abolished in presence of Tetrac (Figure 3). No significant increment was observed in presence of T3 (3 nM) (data not shown).

T3 and T4 effects on HMVEC-C migration

Stimulation with T3 (1 μM) increased cell migration by 1.67 fold (67%) over basal rate of cell migration ($p<0.01$); T4 increased cell migration by 1.50 fold (50%) ($p<0.01$). Incubation with Tetrac had no effect on basal rate, conversely, it abolished migration by T3 ($p<0.01$) and by T4 ($p<0.01$) VEGF (1 μM) increased cell migrationof 60% over basal whereas Tetrac abolished the effect (Figure 4). No significant increment was observed in presence of T3 (3 nM) (data not shown).

Sq RT-PCR analysis

Sq RT-PCR analysis indicates that T3 increased expression of Angpt-1 of 2.5-fold, AAMP 1.6-fold and VEGF 1.2-fold over control, whereas no expression of angiopoietin-2 (Angpt-2) was detected. Only a modest increment by T4 was observed on Angpt-1 1.3-fold and AAMP 1.16-fold over control. Tetrac inhibited mRNA expression of Ang-1, AAMP and VEGF both in control, T3 and T4 samples (from 1.5 to 4-fold) whereas it induced expression of Angpt-2 both in control, T3 and T4 samples (from 4 to 20-fold) (Figure 5).

Discussion

Angiogenesis may be a target for combating diseases characterized by poor or abnormal vasculature, such as ischemic chronic wounds and coronary heart disease, results of failure or insufficient blood vessel formation [3,4].

Thyroid hormones are very important for several physiological events, including growth, development and differentiation [24]. TH are considered strong pro-angiogenic factors, suggesting their potential therapeutic role in cardiac diseases. In the heart, TH genomic actions depend on the interaction with nuclear receptors modulating cardiac myocyte gene expression. Growing evidences support T3 and T4 nongenomic pathways, resulting from their binding to

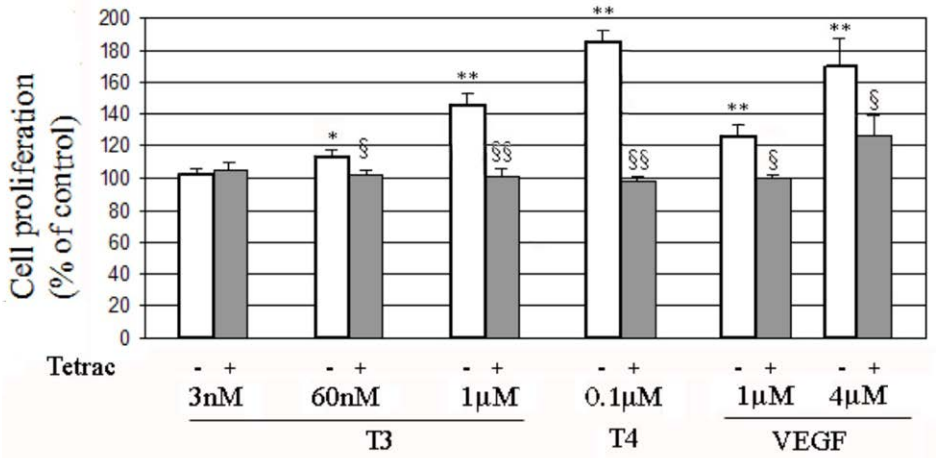

Figure 2: Effect of T3 and T4 on HMVEC-C proliferation. Cells (3 × 10^3 cell/well) were incubated at 48 hrs with T3 (3 nM, 60 nM, 1 μM) or T4 (0.1 μM) alone (open histograms) or in combination with Tetrac (T) (5 μM) (gray histograms). VEGF (1 μM and 4 μM) was assayed as positive control. Data are the mean ± SD expressed as percentage of proliferating treated cells with respect to control cells: *$p<0.05$, **$p<0.01$; §$p<0.05$, §§$p<0.01$: treated cells (T3, T4 or VEGF) with Tetrac vs the corresponding cells without Tetrac.

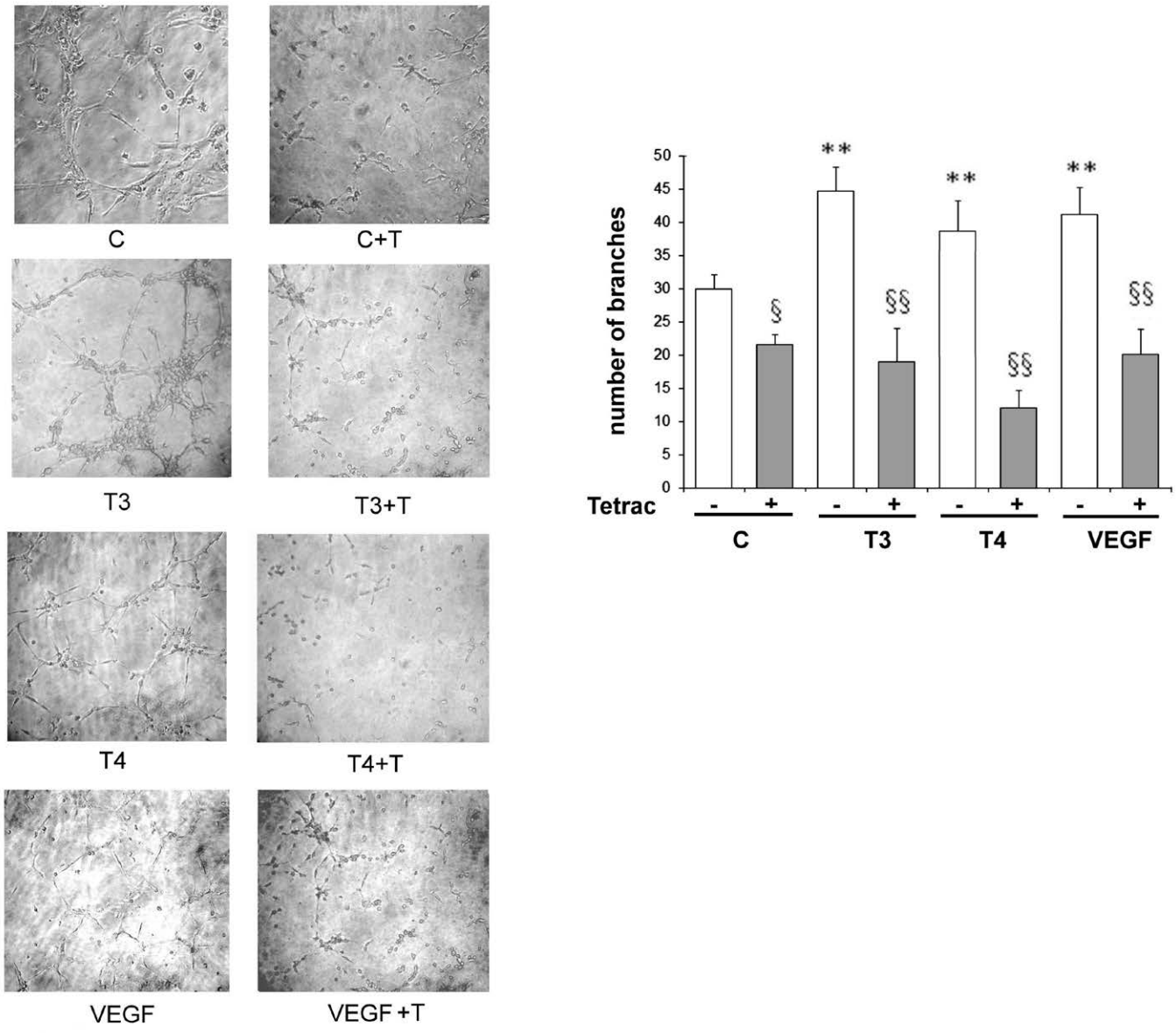

Figure 3: Effect of T3 and T4 on HMVEC-C capillary network formation. HMVEC-C were plated on Matrigel in the absence (C) or presence of T3 (1 μM) and T4 (0.1 μM). VEGF (1 μM) was assayed as positive control. HMVEC-C were also treated with Tetrac (T) (5 μM). The histogram represents the mean value ± SD (seven microscopic fields) of network density. * $p<0.05$, ** $p<0.01$ treated cells with respect to control cells: **§** $p<0.05$, **§§** $p<0.01$: cells (C, T3, T4 or VEGF) with Tetrac vs the corresponding cells without Tetrac.

plasma membrane receptor that leads to a rapid regulation of cardiac functions [25]. TH non-genomic action seems to initiate on plasma membrane integrin αvβ3 receptor, that belongs to the integrin family, heterodimeric cell surface receptors with a pivotal role in cell adesion and migration as well as growth [26,27].

It has been documented that THs promotes new blood vessel formation in models of the chick chorioallantoic membrane (CAM) [22] and tubule formation by human dermal microvascular endothelial cells (HDMEC) [23]. Moreover, it has been found that Tetrac, a T4 analog, inhibited the binding of iodothyronines to the integrin receptor and blocked thyroid hormone effects on angiogenesis and cancer cell proliferation.

In this work we found that integrin β3 receptor is present on plasma membrane of HMVEC-C cells as also reported in other endothelial cell types such as in HUVECs [28] and that treatment with T3 or T4 does not seem to change integrin β3 expression. Furthermore, our study indicates that T3 and T4 directly stimulate angiogenesis in these cells as observed for capillary density, cell proliferation and cell migration. This effect is integrin-mediated because it is blocked in all the considered aspects by Tetrac. T4 seems to be more potent than T3 as stimulator of cell proliferation, whereas they seem equally potent in the other considered angiogenic aspects. At the same time, we confirm that T4 is effective at physiological concentration of 10^{-7} M, whereas T3 is only effective at supraphysiological concentration of 10^{-7} M [13]. In fact, as reported by Bergh et al 2005, integrin αVβ3 appears to bind

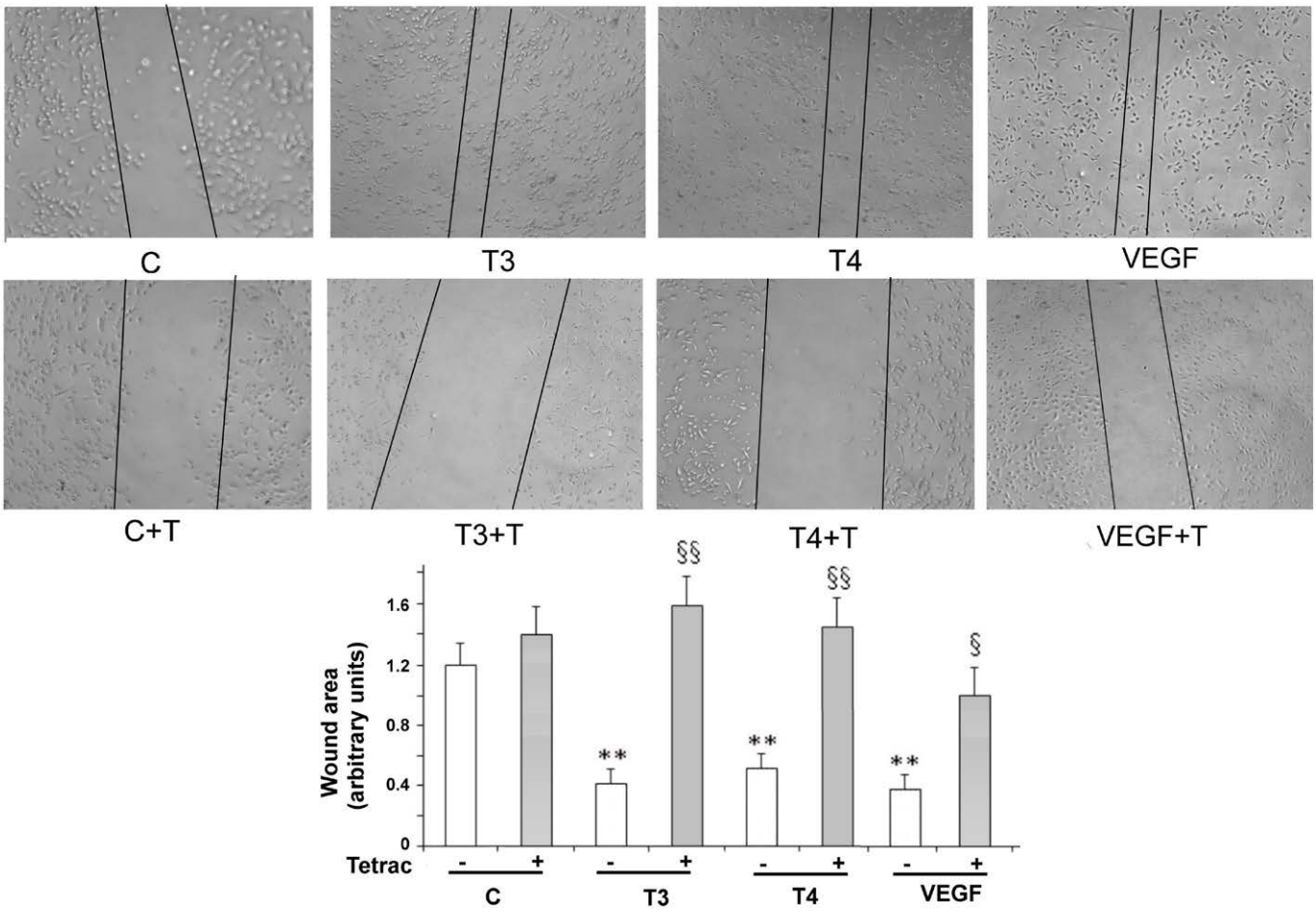

Figure 4: Evaluation of HMVEC-C migration by scratch assay. Data are reported as wounding area (arbitrary units) measured by image analysis. Representative images of healing in basal condition (C) and after stimulation by T3 (1 μM), T4 (1 μM) or VEGF (1 μM) with or without Tetrac (T), after 48 h. The histogram represents the mean value ± SD (six microscopic fields).*p<0.01 treated cells vs basal control; **§**p<0.05, **§§**p<0.01: cells (C, T3, T4 or VEGF) with Tetrac vs the corresponding cells without Tetrac.

T4 rather than T3. This is consistent with previous reports that show mitogen-activated protein kinase (MAPK; ERK1/2) activation and nuclear translocation [29-31] as well as hormone-induced angiogenesis by T4 more than T3. It is known that ERK1/2 plays a central role in cell proliferation. Moreover non-genomic effects, as those starting at the integrin receptor level, may culminate in gene transcription, making more difficult to distinguish when non-genomic or genomic effects of TH do occur. New blood vessel growth in the brain [32] and also in myocardium has been shown after iodothyronine treatment [33]. On the bases of these observations, we can hypothesize that the nongenomic TH actions contribute to cellular events such as angiogenesis and cell proliferation that lead to a rapid regulation of cardiac functions. In our study, we observed also that both T3 and T4 stimulate the expression of some angiogenic-related genes (AAMP, VEGF Angpt-1) confirming a direct effect on angiogenesis. Tetrac reduced the expression of these genes except for Angpt-2 where stimulation by Tetrac was observed both in the untreated control, T3 or T4 treated cells. These data are not in agreement with those reported by Mousa et al. [34] on Tetrac inhibition of mRNA expression of angiopoietin-2 without affecting angiopoietin-1 levels of mRNA. On the other hand it is known that angiopoietin-2 is a negative regulator of Angpt-1 angiogenic factor essential for normal vascular development [35]. Recently Savinova et al. demonstrated that the expression of Angpt-1 in heart positively correlates with TH; conversely, the expression of Angpt-2 was decreased by T3 treatment [36]. Our data seem to indicate a possible role of regulation on Angpt-1 and Angpt-2 by THs and Tetrac in HMVEC-C, but further studies are needed to delineate the mechanism.

Finally our study suggests that Tetrac can inhibit the expression of target genes that are necessary for stimulation of angiogenesis supporting its potential role as anti-tumoral drug.

Taken as a whole, the above reported data indicate the potential angiogenic effect of TH-mediated by integrin aVß3, suggesting that TH could be considered as a pharmacological treatment in situation of non-malignant vascularization, like as in cardiac diseases where a reduction of capillary density has been reported.

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Disclosure Statement

The authors have nothing to disclose.

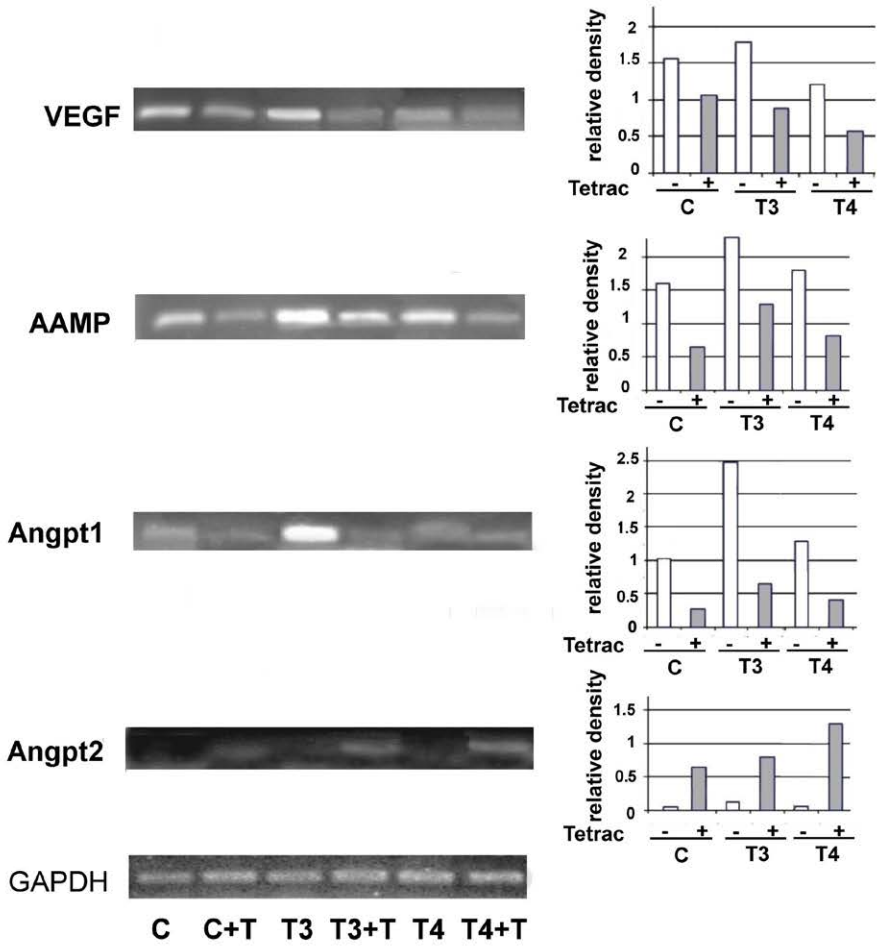

Figure 5: T3, T4 stimulation of angiogenetic-related gene expression in HMVEC-C cells. Sq RT-PCR for AAMP, VEGF, Angpt-1, Angpt-2 in HMVEC-C cells in basal condition or after stimulation by T3 (1 μM), T4 (1 μM) with or without Tetrac (T) for 24 hr. GAPDH was amplified as internal control. The histograms represent the density rations of the angiogenetic-related gene to GAPDH and are representative of three independent experiments.

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