Thyroid hormones promote cell differentiation and up-regulate the expression of the seladin-1 gene in in vitro models of human neuronal precursors

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2002, Santisteban & Bernal 2005). In addition, a time-regulated seladin-1 caspase 3. The aim of this study was to determine whether properties mainly due to the inhibition of the activation of affected by Alzheimer’s disease. Seladin-1 has anti-apoptotic which has been found to be down-regulated in brain regions affected by Alzheimer’s disease. Seladin-1 has anti-apoptotic properties mainly due to the inhibition of the activation of caspase 3. The aim of this study was to determine whether seladin-1 may be regarded as a new mediator of the effects of TH in the developing brain. In order to demonstrate this hypothesis, the effects of TH both on cell differentiation and on the expression of seladin-1 were assessed in two different cell models, i.e. fetal human neuroepithelial cells (FNC) and human mesenchymal stem cells (hMSC), which can be differentiated into neurons. 3,3',5-Triiodothyronine (T3) determined different biological responses (inhibition of cell adhesion, induction of migration, and increase in the expression of the neuronal marker neurofilament-M and Na+ and Ca2+ channel functionality) in both FNC and hMSC, which express TH receptors. Then, we showed that TH significantly increase the expression levels of seladin-1, and that T3 effectively prevents camptothecin-induced apoptosis. However, in hMSC-derived neurons the expression of seladin-1 was not affected by TH. Our results demonstrated for the first time that seladin-1 is a novel TH-regulated gene in neuronal precursors. In view of its anti-apoptotic activity, it might be hypothesized that one of the functions of the increased seladin-1 levels in the developing brain may be to protect neuronal precursor cells from death. Journal of Endocrinology (2008) 197, 437–446

Abstract

Thyroid hormones (TH) play an important role in the development of human brain, by regulating the expression of specific genes. Selective Alzheimer’s disease indicator-1 (seladin-1) is a recently discovered gene with neuroprotective properties, which has been found to be down-regulated in brain regions affected by Alzheimer’s disease. Seladin-1 has anti-apoptotic properties mainly due to the inhibition of the activation of caspase 3. The aim of this study was to determine whether seladin-1 may be regarded as a new mediator of the effects of TH in the developing brain. In order to demonstrate this hypothesis, the effects of TH both on cell differentiation and on the expression of seladin-1 were assessed in two different cell models, i.e. fetal human neuroepithelial cells (FNC) and human mesenchymal stem cells (hMSC), which can be differentiated into neurons. 3,3',5-Triiodothyronine (T3) determined different biological responses (inhibition of cell adhesion, induction of migration, and increase in the expression of the neuronal marker neurofilament-M and Na+ and Ca2+ channel functionality) in both FNC and hMSC, which express TH receptors. Then, we showed that TH significantly increase the expression levels of seladin-1, and that T3 effectively prevents camptothecin-induced apoptosis. However, in hMSC-derived neurons the expression of seladin-1 was not affected by TH. Our results demonstrated for the first time that seladin-1 is a novel TH-regulated gene in neuronal precursors. In view of its anti-apoptotic activity, it might be hypothesized that one of the functions of the increased seladin-1 levels in the developing brain may be to protect neuronal precursor cells from death. Journal of Endocrinology (2008) 197, 437–446

Introduction

Thyroid hormones (TH) play a fundamental role in fetal life, particularly in promoting brain development. TH affect the expression of several genes, which are related to cell migration (i.e. reelin, laminin, tenascin C), myelination (i.e. myelin basic protein, proteolipid protein, myelin-associated glycoprotein), and neuronal differentiation (i.e. nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF); König & Neto 2002, Santisteban & Bernal 2005). In addition, a time-regulated activity of type II and III iodothyronine deiodinases (D2 and 3) in different brain areas is essential during brain development (Kester et al. 2004). Accordingly, it has been shown that early maternal hypothyroxinemia alters fetal brain histogenesis and cytoarchitecture in rats (Lavado-Autric et al. 2003), and unrecognized hypothyroidism in women in the first trimester of pregnancy may adversely affect the neuropsychological development of the progeny (Haddow et al. 1999, Pop et al. 2003, Morreale de Escobar et al. 2004). These observations raised the attention of the scientific community on the benefit of screening programs for assessing thyroid function in pregnant women (Allan et al. 2000, de Escobar 2001, Redmond 2002, Surks et al. 2004).

Selective Alzheimer’s disease indicator-1 (seladin-1) is a gene which has been identified a few years ago and which has been found to be down-regulated in brain regions affected by Alzheimer’s disease (AD). Conversely, in vitro overexpression of seladin-1 conferred protection against β-amyloid-mediated toxicity and from oxidative stress (Greeve et al. 2001, Kuehnle et al. 2008). The neuroprotective effect of seladin-1 appears to be due, at least in part, to the inhibition of caspase 3 activity, a key mediator of apoptosis (Greeve et al. 2001). We have
demonstrated that estrogen and the estrogen receptor modulators, raloxifene and tamoxifen, protect fetal human neuroepithelial cells (FNC) from β-amyloid and H2O2 toxicity by increasing the expression of seladin-1 (Benvenuti et al. 2005, Luciani et al. submitted). These cells were isolated, cloned, and propagated previously from fetal olfactory epithelium (Vannelli et al. 1995). FNC cells show unique features, because they synthesize both neuronal proteins and olfactory markers and respond to odorant stimuli, suggesting their origin from the stem cell compartment that generates mature olfactory receptor neurons. In addition, we have recently demonstrated that high levels of expression of seladin-1 were detectable in human mesenchymal stem cells (hMSC), whereas a significant reduction was observed in hMSC-derived neurons (hMSC-n; Benvenuti et al. 2006). We have also detected high levels of seladin-1 transcript in the adult hippocampus (Benvenuti et al. 2006), one of the brain areas affected in AD (Selkoe 2001), in which stem cells with a defined neurogenic potential are located in the adult brain (Lie et al. 2004). These findings suggest that seladin-1 is a predominant product of multipotent cells and that the defective seladin-1 expression detected in AD-vulnerable brain regions may be linked to an impaired neuronal stem cell compartment, which could be a potential risk factor for the development of this disease. Interestingly, there is evidence both in embryonic and in adult mammals that TH plays a key role in the development and maintenance of basal forebrain cholinergic neurons typically involved in AD (Patel et al. 1987, Calzà et al. 1997).

Therefore, we hypothesized that seladin-1 may be one of the mediators of the effects of TH during the development of the nervous system. According to this hypothesis, in the present study, we first determined the role of TH on cell differentiation in FNC and hMSC, by addressing their effects on cell adhesion, invasiveness, expression of the neuronal marker neurofilament-M, Na+ and Ca2+ channels functionality as evaluated by their current density, and activation and inactivation voltage dependence. Thereafter, we analyzed whether TH affect the expression of seladin-1 in these cells and prevent apoptosis. In order to clarify whether seladin-1 may be targeted as a TH-regulated gene selectively in neuronal precursors, the effect of TH on seladin-1 expression was also determined in hMSC-n.

Materials and Methods

Materials

Media and sera for cell cultures were purchased from Euroclone (Wetherby, West York, UK) and tissue plasticware was obtained from GreinerBio-One (Frickenhausen, Germany). The neurobasal medium was obtained from Life Technologies (Invitrogen). Other reagents for cell cultures were obtained from Sigma. The flow cytometry buffer (CellWASH) was obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Monoclonal antibodies for flow cytometry immunophenotyping were obtained from BD Pharmingen (San Diego, CA, USA) and Ancell (St N Bayport, MN, USA), whereas antibodies for immunocytochemistry were from Chemicon (Temecula, CA, USA). For RNA extraction, the Nucleospin RNAII kit was purchased from Macherey-Nagel (Duren, Germany). Reagents for RT-PCR studies were from Applied Biosystem Inc. (Foster City, CA, USA). TH (3,3′,5-triiodothyronine, T3 and 1-thyroxin, T4) and camptothecin were purchased from Sigma. Molecular weight marker GeneRuler 100 and 50 bp DNA Ladder were from Fermentas (Burlington, Ontario, Canada).

Cell cultures

FNCs were isolated from human fetal olfactory neuroepithelium, as described previously (Vannelli et al. 1995). Long-term FNC cell cultures were propagated in Coon’s-modified Ham’s F12, supplemented with 10% fetal calf serum (FCS) and antibiotics (growth medium). The B4 clone, showing the highest level of the expression of neuronal and olfactory markers, was used in this study. hMSC were isolated from the iliac crest of normal donor marrow aspirates. Informed consent was obtained from all donors and the institutional ethical committee approved all the procedures. hMSC were isolated and characterized as described previously (Benvenuti et al. 2006). The induction of a neuronal phenotype in hMSC was performed according to the method of Woodbury et al. (2000), with some modifications, as described previously (Benvenuti et al. 2006).

RT-PCR for TH receptors (TR) and type I and II deiodinases (D1 and D2)

Total RNA was isolated from FNC, hMSC, and hMSC-n. RT-PCR was performed using SuperScript III One-Step RT-PCR with Platinum Taq kit (Invitrogen Life Technologies Inc.) with 1 μg total RNA as template. Specific oligonucleotides were used as upstream and downstream primers, as described previously (Gittoes et al. 1997, de Souza Meyer et al. 2005). All reactions were performed in duplicate. After amplification, 10 μl aliquots of the PCR products were analyzed on a 2% ethidium bromide agarose gel and visualized under u.v. light using Kodak Image Station 140 CF.

Cell adhesion assay

For cell adhesion assay, the cells were plated in 96-well plates at a density of 5×103 (hMSC) cells/well and 6×103 (FNC) cells/well with or without 1 nM T3 in medium with 1% FCS for 24 h. At the end of the incubation, the cell monolayer was washed twice with PBS in order to remove the non-adherent cells by aspiration and 100 μl Rose Bengal stain (0-25% in PBS) was added to each well for 5 min at room temperature (Gamble & Vadas 1988). After aspiration of the stain, each well was washed twice in PBS and the stain was released by adding 200 μl/well of an ethanol/PBS (1:1) solution for 30 min. The samples were analyzed using an ELISA plate
were expressed as mean \( \pm \) S.E.M. The experiments were performed in hexaplicate and were repeated at least three times.

**Quantitative cell migration assay**

hMSC and FNC cells were passaged twice prior to the assay and were washed with PBS. Successively, the cells were detached using a harvesting buffer (0.05% trypsin in Hank’s balanced salt solution containing 25 mM HEPES), and a quenching medium (serum-free Dulbecco’s modified minimum essential medium (DMEM) containing 5% BSA) was added to the dishes. After centrifugation, the cells were resuspended in the quenching medium and 500 \( \mu \)l cell suspension, with or without 1 nM T3, was added to both collagen I- and BSA-coated Boyden chambers (top). The same number of cells was plated in collagen I-coated wells for cell adhesion and morphology control. After 20 h incubation, the cells were stained using crystal violet according to the manufacturer’s instructions. Finally, 300 \( \mu \)l extraction buffer was added to each membrane for 15 min, then a 10 \( \mu \)l stained solution was removed and placed in 96-multiwell plates, and the absorbance was read at 540 nm.

**Immunocytochemistry**

hMSC were cultured in four-chamber slides in a growth medium. Subsequently, the cells were treated with 1 nM T3 for 4 days in a serum-free medium. Then, they were washed twice with PBS, fixed in 4% paraformaldehyde and 0.1% glutaraldehyde, and were immunostained for neurofilament-M (NF-M) using a rabbit anti-NF-M C-terminal antibody obtained as reported previously (Luciani et al. 2004). The cells were then washed twice with PBS, fixed in 4% paraformaldehyde and 0.1% glutaraldehyde, and were immunostained for neurofilament-M using a rabbit anti-NF-M C-terminal antibody obtained as reported previously (Luciani et al. 2004). Finally, the cells were exposed to Vectastain ABC and AEC reagents (Vector Laboratories Inc., Burlingame, CA, USA) and counterstained with hematoxylin. Immunopositive cells/field were counted per experiment \( (n=3) \) and the results were expressed as mean \( \pm \) S.E.M.

**Electrophysiological studies**

The electrophysiological records were obtained on FNC and hMSC (control and T3-treated) adherent on glass cover slips using the whole-cell patch-clamp technique under voltage-clamp conditions. The experimental setup, microelectrodes, pulse protocols of stimulation, data acquisitions, and analysis were as previously described in detail (Benvenuti et al. 2006). When outward \( K^+ \) currents had to be suppressed, parallel experiments were performed using a 20 mM TEA solution containing 122.5 mM NaCl, 2 mM CaCl\(_2\), 20 mM TEA-OH, and 10 mM HEPES. To test the high-voltage-activated \( \text{Na}^+ \) channels’ sensitivity, TTX (1 \( \mu \)M) was used. In order to have \( \text{Ca}^{2+} \) as the only permeant cation, \( \text{Ca}^{2+} \) currents were recorded in a TEA-\( \text{Ca}^{2+} \) bath solution, 10 mM CaCl\(_2\), 145 mM TEABr, and 10 mM HEPES. In turn, 10 \( \mu \)M nifedipine or 100 \( \mu \)M Cd\(^{2+}\), added as CdSO\(_4\), were used to block L-type \( \text{Ca}^{2+} \) channels and all but T-type \( \text{Ca}^{2+} \) channels respectively. The steady-state ionic current activation was evaluated using

\[
I_s(V) = G_{\text{max}}(V-V_{\text{rev}})[1 + \exp((V_s - V)/k_s)]
\]

and the steady-state inactivation using

\[
I_h(V) = I_h[1 + \exp(-(V_h - V)/k_h)]
\]

where \( G_{\text{max}} \) is the maximal conductance for \( I_s \), \( V_{\text{rev}} \) is the apparent reversal potential, \( V_s \) and \( V_h \) are the potentials that elicit the half-maximal size, and \( k_s \) and \( k_h \) are the steepness factors. For the activation curve evaluation, the cell was held at \(-80\) mV and step pulses (10 ms or 1 s for \( \text{Na}^+ \) current, \( I_{\text{Na}} \), and \( \text{Ca}^{2+} \) current, \( I_{\text{Ca}} \) recording respectively) ranging from \(-70\) to 50 mV were applied in increments of 10 mV. The inactivation curve was evaluated on the current elicited by a voltage step at 0 mV, pre-pulsed by voltage steps as those used for activation with an inter-pulse period of 2 or 20 ms for \( I_{\text{Na}} \) and \( I_{\text{Ca}} \) recording respectively. Data are expressed as mean \( \pm \) S.E.M.

**Quantitative real-time RT-PCR**

The quantification of seladin-1, integrin subunits \( \alpha V, \beta 3 \), and \( \alpha 2b \) mRNA, was performed by real-time RT-PCR, based on TaqMan technologies. The total RNA to be subjected to RT was extracted from FNC, hMSC, and hMSC-n (basal conditions and after T3 or T4 treatment for 72 h in serum-free medium). Total RNA isolation and cDNA synthesis were obtained as reported previously (Luciani et al. 2004). The primers and probe for the integrin subunits \( \alpha V, \beta 3 \), and \( \alpha 2b \) were Assay-On-Demand gene expression products (Applied Biosystems); the PCR mixture (25 \( \mu \)l final volume) consisted of \( 1 \times \) final concentration of Assay-On-Demand mix, \( 1 \times \) final concentration of Universal PCR Master Mix (Applied Biosystems), and 20 ng cDNA. Each measurement was carried out in triplicates and three experiments were performed. The mRNA quantification was based on the comparative \( C_{\text{t}} \) method according to the manufacturer’s instructions (Applied Biosystems) and data were normalized to ribosomal 18S RNA expression. The results were expressed as percentage mRNA versus control. The conditions used for seladin-1 mRNA measurement were described previously (Luciani et al. 2004).

**TUNEL analysis for the determination of apoptotic cells**

Apoptosis was determined by TUNEL analysis, using a commercially available detection kit (FragEL DNA Fragmentation Detection Kit, Oncogene Research Products, Boston, MA, USA), following the manufacturer’s instructions. Briefly, FNC and hMSC were cultured in chamber slides both in the presence and in the absence of 1 nM T3 for 72 h. During the last 4 h, one half of T3-treated and the other half of untreated
chamber slides were exposed to 20 μg/ml camptothecin. Three experiments were performed. The number of apoptotic cells/field was counted in ten 20× fields for each experiment and the results were expressed as percentage of apoptotic cells/field (mean ± s.e.m).

Results

Expression of TR in FNC and hMSC

The presence of transcripts corresponding to TRα1, β1, and β2 (i.e. the major TR isoforms with a known biological role) in FNC and hMSC was evaluated by RT-PCR. In both the cases, detectable amounts of mRNA of these TR isoforms were found, as shown in Fig. 1.

Effect of T3 on cell adhesion, migration, and expression of NF-M

No study has so far addressed the effect of TH in promoting the neuronal differentiation of FNC and hMSC. Therefore, the first objective of this study was to determine whether T3

Figure 1 Detection of TR isoforms α1, β1, and β2 mRNA by RT-PCR in FNC and hMSC. C+, positive control, i.e. normal thyroid tissue. MW100 and MW50, molecular weight marker (see Materials and Methods).

Figure 2 (A) Effect of T3 (1 nM) on cell adhesion. (B) Amount of integrin subunits αV, β3, and α2β1 mRNA in FNC and hMSC, without or with T3 exposure. C, control, i.e. absence of T3 exposure. *P<0·05 vs C.

Figure 3 Effect of T3 (1 nM) on cell migration in FNC and hMSC. A typical experiment is shown in (A), and the quantitative analysis is represented in (B).
induces biological responses, which are expected in differentiating cells, in our particular cell models. First, the effect of TH on cell adhesion was determined. The exposure to T₃ (1 nM) for 24 h induced a significant inhibition of cell adhesion in FNC as well as in hMSC (Fig. 2A). Accordingly, the expression of integrin subunits α2β₁, β₃ and, in the case of hMSC, also αV was significantly reduced by T₃ exposure (Fig. 2B). With regard to cell migration, exposure to 1 nM T₃ determined a significant stimulatory effect in both the cell models (Fig. 3A and B). Immunocytochemistry for the neuronal marker NF-M was also performed. In untreated FNC, positivity for NF-M was rarely observed, whereas T₃-treated cells showed a strong positivity, decorating specifically both the cell body and the neurite-like processes (Fig. 4A) (8 ± 1.1 vs 49.7 ± 6.9 cells/field, mean ± S.E.M., P < 0.005). Similarly, in untreated hMSC a faint positivity was observed in a few cells. Upon T₃ exposure, the intensity of the staining and the number of positive cells markedly increased (Fig. 4B) (13.2 ± 0.9 vs 41.4 ± 3.6 cells/field, mean ± S.E.M., P < 0.05).

Effects of T₃ on the electrophysiological properties of FNC and hMSC

Based on previous evidence indicating a positive effect of T₃ on neuron excitability (Hoffmann & Dietzel 2004), the effect of T₃ on the electrophysiological properties of FNC and hMSC was also assessed. All control FNC exhibited Iₐss, as shown in a typical experiment displayed in Fig. 5A. The voltage threshold of Iₐss was about −50 mV. The treatment with T₃ (1 nM for 72 h) definitely increased Iₐss amplitude (Fig. 5B) and this was consistently observed in all the experiments. The normalized I–V plot determined at the current peak related to all the cells investigated is shown in Fig. 5C. With regard to hMSC, these cells usually did not show a typical voltage-activated Iₐss. Only a low percentage (<5%) of hMSC cultured on the glass cover slips for 3 days showed Iₐss with a very small amplitude, but the 50% of hMSC cultured on the glass cover slips for 6 days showed Iₐss (Fig. 5E). Again, the treatment with T₃ (1 nM for 6 days) determined an enhancement of Iₐss (Fig. 5F) and increased the number of responsive cells to 80%. The normalized I–V plot related to all the cells investigated is shown in Fig. 5G.

Figure 4 Immunostaining for NF-M in (A) FNC and (B) hMSC in basal conditions or after exposure to T₃.

Magnification 40×.
The maximal current amplitude was recorded at 0 mV in control and T3-treated hMSC, as well as in control FNC but it was at $-10$ mV in FNC treated with T3 (Fig. 5A–C and E–G). Interestingly, the time to peak decreased in T3-treated cells (Fig. 5A, B, and E, F). Moreover, the Boltzmann parameters underwent a change because of T3 treatment. The half voltage activation parameter, $V_a$, was negatively shifted to $\sim 5$ (FCN) and $9$ (hMSC) mV in T3-treated cells with respect to the control (Fig. 5D and H; Table 1). The T3 treatment determined a decrease in the $k_a$ values, which was not significant in FNC but was significant in hMSC ($P<0.05$) (Table 1). $k_a$, inactivation in T3-treated FNC did not show any significant change with respect to control, whereas in hMSC a $7$ mV significant shift ($P<0.05$) towards more positive potentials was observed for $V_a$. The T3 treatment did not determine a significant reduction in $k_i$, in both FNC and hMSC (Fig. 5D and H; Table 1). In conclusion, T3 treatment determined a fast kinetics of $I_{Na}$, and changed the $V_a$ Boltzmann parameters for activation in both FNC and hMSC. Such changes were more evident in the less-differentiated hMSC than in FNC, in which, together with $V_a$, the T3 treatment also affected $k_a$ and the $V_h$ Boltzmann parameters for inactivation.

All control FNC exhibited $I_{Ca}$ (Fig. 6A). The treatment with T3 (1 nM for 72 h) definitely increased $I_{Ca}$ amplitude (Fig. 6B) and this was consistently observed in all the experiments. The normalized I–V plot related to all the cells investigated is shown in Fig. 6C. $I_{Ca}$ was the result of T-type (not blocked by nifedipine or Cd$^{2+}$) and L-type $I_{Ca}$ (blocked by nifedipine or Cd$^{2+}$). The voltage that elicited the maximal L-type $I_{Ca}$ was shifted towards $\sim 10$ mV mV more negative potentials, and the related time to peak was reduced by $\sim 100$ ms in T3-treated cells. The related Boltzmann parameters for T-type $I_{Ca}$ activation were not changed by T3 treatment (Fig. 6D; Table 1). In contrast, the parameters for L-type $I_{Ca}$ activation underwent a change as a result of T3 treatment (Fig. 6D; Table 1); the values of $V_a$ were shifted to $\sim 7$ mV towards more negative potentials ($P<0.05$) and those for $k_a$ were significantly reduced ($P<0.05$). Again, T-type $I_{Ca}$ inactivation in T3-treated FNC also did not show any significant change with respect to the control, whereas the L-type $I_{Ca}$ showed a $4$ mV significant shift ($P<0.05$) towards more negative potentials (Fig. 6D). The T3 treatment did not determine significant changes in $k_i$. In conclusion, T3 treatment caused in FNC a fast kinetics of L-type $I_{Ca}$, and changed the $V_a$ and $V_h$ Boltzmann parameters for activation and inactivation respectively as observed for $I_{Na}$. With regard to hMSC, in both control and T3-treated cells, $I_{Ca}$ was not observed, or its size was very small respect to the noise and therefore not accurately measurable (not shown).

**Effect of TH on seladin-1 expression and on apoptosis**

The effect of TH on the expression of *seladin-1* was assessed by real-time RT-PCR. Interestingly, T3 (1 nM) and, yet to a lower extent, T4 (1 nM) determined a significant increase of *seladin-1* mRNA in FNC (325 ± 7.5 fg/μg total RNA, T3; 225 ±
Table 1 Effect of 3,3',5-triiodothyronine (T₃) treatment on Boltzmann parameters of activation and inactivation curves for Iₒa in fetal human neuroepithelial cells (FNC) and in 6 days cultured human mesenchymal stem cells (hMSC) and for Iₒa in FNC.

<table>
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<th>FNC</th>
<th>hMSC</th>
<th>Iₒa (FNC)</th>
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<tr>
<td></td>
<td>Con</td>
<td>T₃</td>
<td>Con</td>
</tr>
<tr>
<td>Vₒ</td>
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<td>-57.2±4.8</td>
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<tr>
<td>kₖ</td>
<td>7.8±0.6</td>
<td>7.5±0.8</td>
<td>8.5±0.6</td>
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*P<0.05 and †P<0.01 versus the corresponding control.

5.3 fg/μg total RNA, T₄ (mean±S.E.M.) and in hMSC (270±6.4 fg/μg total RNA, T₃; 195±4.8 fg/μg total RNA, T₄), when compared with the basal amount of transcript (135±2.8 fg/μg total RNA in FNC; 167±3.34 fg/μg total RNA in hMSC) (Fig. 7A). Furthermore, in agreement with previous studies associating the induction of seladin-1 expression to anti-apoptotic properties (Greeve et al. 2001, Benvenuti et al. 2005, Kuehnle et al. 2008), we found that T₃ (1 nM) was able to significantly counteract camptothecin-induced apoptosis, as determined by TUNEL analysis, and in the case of FNC the percentage of apoptotic cells after camptothecin and T₃ treatment was even superimposable with that of control cells (Table 2).

In order to determine whether the effect of T₄ was mediated by its conversion into T₃, deiodinase (D1 and D2) expression was determined in FNC and hMSC by RT-PCR. Whereas no detectable amount of D1 transcript could be found in these cells, D2 transcript was present in both FNC and hMSC, as shown in Fig. 7B, thus suggesting that the stimulatory effect of T₄ on the expression of seladin-1 is, at least partially, due to the intracellular conversion into T₃.

In order to clarify whether seladin-1 may be targeted as a TH-regulated gene selectively in in vitro models of multipotent undifferentiated cells, the effect of TH on seladin-1 expression was also determined in differentiated neurons. For this purpose, hMSC were differentiated into a neuronal phenotype (hMSC-n) and proven to be neurons, as described previously (Benvenuti et al. 2006). In hMSC-n, the expression of detectable levels of TRα1, β1, and β2 was maintained (Fig. 8A). Noteworthy, the amount of seladin-1 mRNA in hMSC-n was significantly lower (25±0.5 fg/μg total RNA) compared with both FNC and hMSC, thus confirming our previous finding that seladin-1 is a predominant product of undifferentiated cells (Benvenuti et al. 2006). Furthermore, neither T₃ nor T₄ was able to modify the level of transcript in hMSC-n (23±5±1.2 fg/μg total RNA, T₃; 24±0±8 fg/μg total RNA, T₄) (Fig. 8B).

Discussion

It is well known that TH play a fundamental role in fetal life, particularly in promoting early brain development, by affecting the expression of specific genes (König & Neto 2002, Santisteban & Bernal 2005).
The numbers represent apoptotic/total cells (mean ± S.E.M.) in ten fields, magnification 20×.

Table 2 Effect of 3,3′,5-triiodothyronine (T3) (1 nM) treatment on camptothecin-induced apoptosis in fetal human neuroepithelial cells (FNC) and human mesenchymal stem cells (hMSC), as determined by TUNEL analysis. The numbers represent apoptotic/total cells (mean % ± S.E.M.) in ten fields, magnification 20×.

<table>
<thead>
<tr>
<th></th>
<th>FNC</th>
<th>hMSC</th>
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<tr>
<td>Control</td>
<td>3 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>T3</td>
<td>4 ± 0.9</td>
<td>2 ± 0.6</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>15±9±2±4*</td>
<td>18±1±1±4*</td>
</tr>
<tr>
<td>Camptothecin+T3</td>
<td>3±6±0.9*</td>
<td>7±5±1±6*</td>
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*P<0.05 versus control, †P<0.05 versus camptothecin.

Figure 7 (A) Measurement of seladin-1 mRNA by real-time RT-PCR in FNC and hMSC, in the absence (C) or in the presence of TH (T1 and T4). *P<0.05 vs C. (B) Detection of D1 and D2 mRNA in FNC and hMSC, as assessed by RT-PCR. C+, positive control, i.e. normal thyroid tissue. MW100, molecular weight marker (see Materials and Methods).

In our study, we used two different cell models, FNC and hMSC. FNC are human neuronal precursors, which generate mature olfactory receptor neurons (Vannelli et al. 1995). The choice of hMSC was based on the fact that they are much more easily obtainable than neuronal stem cells, and we rationalized that they might represent an acceptable alternative to the use of neuronal stem cells in our experimental setting. In fact, in vitro and in vivo neuronal differentiation of MSC into neurons has been demonstrated (Woodbury et al. 2000, Sanchez-Ramos 2002, Benvenuti et al. 2006). In addition, transplanted bone marrow cells have been localized in mouse (Calza et al. 1997, Mezey et al. 2000) and human brain (Mezey et al. 2003, Weimann et al. 2003), suggesting a possible differentiation into neuronal cells. Accordingly, the functional/structural parallelism between neuronal stem cells and MSC has been underlined by the proposal to use terms like ‘neuropoiesis’ to indicate the persistence of neurogenesis in the adult brain and ‘brain marrow’ to describe the brain regions that contain the cells supporting neurogenesis (Steindler et al. 1996, Scheffer et al. 1999).

We demonstrated that in both FNC and hMSC, which express TRα1, B1, and B2, T3 (1 nM) was able to reduce cell adhesion. This finding was paralleled by the reduced expression of adhesion molecules, such as different α and β integrin subunits. In addition, T3 increased cell migration, in agreement with similar findings obtained in different cell models (König & Neto 2002, Santisteban & Bernal 2005). Furthermore, an increase in both the intensity of the immunostaining and in the number of positive cells for NF-M, a typical neuronal marker, was observed in hMSC and in FNC, following T3 exposure. Electrophysiological evaluation revealed that in these cell models, there were positive effects on Na+ and L-type Ca2+ channels upon T3 treatment. T3 induced a faster activation kinetics on IksNa, as indicated by the reduced time to peak, increased its current density, and shifted its activation and inactivation curves towards more negative and more positive potentials respectively. Such changes, which were more evident in hMSC than in FNC, indicated that T3 was able to induce the expression of new functional ionic channels and suggested a permissive role towards the differentiation into a proper excitable neuronal phenotype. T3 had similar effects on L-type Ca2+ channels expressed in FNC; it reduced L-type ICa time to peak, increased its current density and shifted its activation and inactivation curves towards more negative and more positive potentials respectively. Thus, T3 not only increased the IksNa and L-type ICa, but also their functionality by inducing faster kinetics and changing their voltage dependence towards a more functional state. In fact, these currents activated at less negative membrane potentials and inactivated at more positive potentials. All these observations clearly indicated that T3 had biological effects on Na+ and L-type Ca2+ channels in FNC, which were already committed towards neuronal differentiation. These effects were more evident on Na+ channels in the lessDifferentiated hMSC, in agreement with the well-established role of TH in promoting neuronal development.

Furthermore, we determined whether TH affect the expression of seladin-1. We found that T3, and to a lesser extent T4, significantly increased the amount of seladin-1 mRNA in both the cell models. The fact that FNC as well as hMSC express type II deiodinase suggests that the effect of T4 on seladin-1 expression may be mainly due to its intracellular transformation into T3. Furthermore, in agreement with previous reports indicating that the up-regulation of seladin-1 expression, such as that obtained for instance upon estrogen exposure, is paralleled by an inhibitory effect on apoptosis (Greeve et al. 2001, Benvenuti et al. 2005), we demonstrated here that T3 also, in addition to its stimulatory role on seladin-1 expression, was able to significantly counteract the induction of apoptosis by camptothecin. These data suggest that seladin-1 may be a mediator of the effects that TH have in the brain.

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According to the knowledge that TH play an essential role in the CN at the time of brain development, we questioned whether their stimulatory effect on seladin-1 expression was maintained or not in differentiated neuronal cells. Thus, we differentiated hMSC toward a neuronal phenotype (hMSC-n), according to an experimental procedure validated previously by us as well as by other groups (Woodbury et al. 2000, Benvenuti et al. 2006). Admittedly, a comparison made using the same cell model at different stages of differentiation would provide striking evidence on whether or not the expression of a gene (i.e. seladin-1) is dependent on the degree of differentiation. In hMSC-n, the amount of expression of seladin-1 was significantly lower than that in hMSC, according to our previous findings (Benvenuti et al. 2006). Noteworthy, neither T4 nor T3 affected seladin-1 expression, thus indicating clearly that the stimulatory effect of TH appears to be selectively present in neuronal precursor cells. This finding is in keeping with observations also from in vivo studies in experimental animals that most of the TH-regulated genes are responsive only during a limited time interval of brain development (König & Neto 2002). The molecular reason for the absent modulation of the expression of the seladin-1 gene by TH in hMSC-n, which express TR, remains elusive at present. However, the expression of TR does not necessarily correlate with the effects of TH. A good example is provided by the observation that in mice lacking the TRα1, which is the predominant TR isoform in the fetal cerebellum, no structural alteration of this organ was found (Morte et al. 2002). On the other hand, the presence of TR does not warrant their function on gene expression, which is the result of a complex series of events, including for instance the association with co-activators or co-repressors (Zhang & Lazar 2000). Finally, the presence of membrane-regulated actions of TH has been described (Davis et al. 2005), and this possibility has been further highlighted by the recent demonstration of a TH cell surface receptor on the extracellular domain of integrin αVβ3, a structural membrane protein (Bergh et al. 2005).

The biological role played by the TH-mediated stimulation of seladin-1 expression in neuronal precursors remains to be elucidated. For this purpose, additional studies, designed for instance to make seladin-1 expression in these cells silent, will be performed, in order to determine which of the TH-mediated function/s is/are directly due to this protein. However, in consideration of the fact that one of the best characterized properties of seladin-1 so far is its anti-apoptotic activity (Greeve et al. 2001, Kuehnle et al. 2008), which has been further confirmed also by our group in FNC cells (Benvenuti et al. 2005, Luciani et al., submitted), it might be hypothesized that one of the functions associated with the increased seladin-1 levels in the developing brain may be to protect neuronal precursor cells from death. From this point of view, seladin-1 might therefore be regarded as a factor, which helps in maintaining a pool of young and self-renewing multipotent cells, to then be made available to other TH-regulated genes, which specifically direct the differentiation toward a neuronal phenotype.

In summary, our study showed for the first time that seladin-1 is a novel TH-regulated gene in neuronal precursors and suggested that it might be an additional mediator of the effects of TH in the developing brain.

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