See related commentary on pg 7

ORIGINAL ARTICLE

TSH Receptor and Thyroid-Specific Gene Expression in Human Skin

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Experimental evidence suggests that in autoimmune thyroid diseases (AITDs) the skin is a target of autoantibodies against thyroid-specific antigens; however, the role of these autoantibodies in skin alterations remains unclear. To gain insight into the function of nominally thyroid-specific genes in skin, we analyzed the expression of thyroid-stimulating hormone–receptor (TSH-R), thyroglobulin (Tg), sodium iodide symporter (NIS), and thyroperoxidase (TPO) genes in normal human skin biopsies and cultured primary keratinocytes and dermal fibroblasts. The results revealed the presence of all the transcripts in skin biopsies. However, in keratinocytes and fibroblasts, only TSH-R messenger RNA was always detected. Western blot and immunohistochemical analyses of skin specimens confirmed the presence of TSH-R protein in keratinocytes and fibroblasts. Moreover, TSH treatment induced the proliferation of cultured keratinocytes and fibroblasts and increased keratinocyte intracellular cAMP. Finally, affinity-purified IgGs from serum of patients affected by Graves' disease, but not by chronic lymphocytic thyroiditis, stimulated cAMP accumulation in cultured keratinocytes, as well as their proliferation. In conclusion, the expression of thyroid-specific genes in cultured keratinocytes and fibroblasts and the mitogenic effects of TSH and IgGs on these cells support the concept that autoantibodies against thyroid-specific antigens may contribute to cutaneous symptoms in AITDs.

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INTRODUCTION

Autoimmune thyroid diseases (AITDs) are the most common organ-specific autoimmune diseases, affecting about 5% of the population. They comprise several pathological conditions, the most common being Graves' disease (GD) and chronic lymphocytic thyroiditis (CLT) (Stassi and De Maria, 2002; Weetman, 2003; Jacobson and Tomer, 2007). Patients affected by AITDs present cellular and humoral immune responses aberrantly directed against the thyroid. Both GD and CLT are characterized by the infiltration of the thyroid gland by B and T lymphocytes and by the production of autoantibodies against thyroid-specific antigens such as thyroglobulin (Tg), thyroperoxidase (TPO), and thyroid-stimulating hormone receptor (TSH-R). In patients affected by GD, the presence of autoantibodies stimulating the TSH-R is responsible for the clinical manifestations of hyperthyroidism, whereas in patients affected by CTL, the inflammatory infiltrate progressively replaces normal parenchyma, resulting in thyrocyte depletion through apoptosis and leading to hypothyroidism and ultimately to gland fibrosis (Stassi and De Maria, 2002; Weetman, 2003).

Skin development and functions are known to be regulated by thyroid hormones, and alterations in skin architecture and homeostasis subsequent to thyroid gland dysfunctions have been documented (Leonhardt and Heymann, 2002; Burman and McKinley-Grant, 2006). In particular, thyroid hormones have been shown to affect fetal epidermal differentiation, barrier formation, hair growth, sebum production, wound healing, epidermal oxygen consumption, keratinocyte proliferation, and keratin gene expression (Hanley *et al.*, 1996; Tomić-Canić *et al.*, 1996; Kömüves *et al.*, 1998; Slominski and Wortsman, 2000; Leonhardt and Heymann, 2002; Radoja *et al.*, 2004; Safer *et al.*, 2004). In accordance with these findings, thyroid hormone receptor expression has been shown to occur in different skin cell types, including keratinocytes, dermal fibroblasts, and sebaceous gland cells. The activity of both type II and type III thyroid hormone

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Abbreviations: AITD, autoimmune thyroid diseases; CLT, chronic lymphocytic thyroiditis; GD, Graves' disease; NIS, sodium iodide symporter; Tg, thyroglobulin; TPO, thyroperoxidase; TSH, thyroid-stimulating hormone; TSH-R, thyroid-stimulating hormone receptor

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deiodinases, the enzymes responsible for thyroid hormone metabolism, has also been detected in human skin (Refetoff *et al.*, 1972; Huang *et al.*, 1985; Billoni *et al.*, 2000; Torma *et al.*, 2000; Dentice *et al.*, 2007).

Patients affected by hypothyroidism are characterized by generalized myxedema—the original term for hypothyroidism (Horsley, 1885)—with cool, dry, and pale skin. In hyperthyroidism, by contrast, the skin is warm and smooth, and a generalized hyperhidrosis may be present (Leonhardt and Heymann, 2002). The dermatological manifestations of hyper- or hypothyroidism are thought to be consequent to alterations of general metabolism (for example, a reduced core body temperature and the resulting cutaneous vasoconstriction are responsible for the cool skin observed in hypothyroidism) and thyroid hormone action on the various skin cell types (Leonhardt and Heymann, 2002; Burman and McKinley-Grant, 2006). In patients affected by AITDs, however, not all skin alterations may be explained exclusively by the variations in circulating thyroid hormones (Ai *et al.*, 2003; Burman and McKinley-Grant, 2006). For example, pretibial myxedema, which occurs in approximately 4–5% of patients affected by GD, is observed in both hyper- and euthyroid patients. Although the pathogenesis of pretibial myxedema remains to be defined, the identification of TSH-R transcripts in fibroblasts and the ability of both TSH and TSH-R antibodies to bind fibroblasts have led to the hypothesis that pretibial fibroblasts may be stimulated by thyroid autoantibodies, resulting in an excessive production of glycosaminoglycans (Wu *et al.*, 1996; Rapaport *et al.*, 2000; Leonhardt and Heymann, 2002). The presence of specific TSH-R transcripts and other nominally thyroid-specific genes, such as the sodium iodide symporter (NIS), a transmembrane glycoprotein responsible in thyrocytes for the accumulation of iodide required for thyroid hormone biosynthesis, and Tg, has also been reported, not only in human dermal fibroblasts but also in keratinocytes, melanocytes, and melanoma cells (Slominski *et al.*, 2002). Moreover, very recently, TSH-R messenger RNA (mRNA) and protein were also detected in human scalp hair follicles, which show differential gene expression after TSH treatment (Bodó *et al.*, 2009). Together, these observations suggest that in the course of AITDs the functions of the different skin cell types may be affected not only by variation in thyroid hormone levels but also by the presence of thyroid-specific autoantibodies.

In this study, to gain more insight into the role of thyroid-specific molecules in human skin, we first analyzed the expression of TSH-R, Tg, NIS, and TPO in normal human skin biopsies and primary cultures of keratinocytes and dermal fibroblasts. We then evaluated whether TSH and IgGs purified from the serum of patients affected by AITDs could exert biological effects on cultured primary human keratinocytes and fibroblasts.

RESULTS

Messenger RNA level of thyroid-specific genes in human skin samples from healthy individuals and cultured primary keratinocytes and fibroblasts

We initially analyzed by means of RT-PCR the expression of the TSH-R, Tg, TPO, and NIS on total RNA extracted from three different human skin biopsies from healthy individuals. The presence of specific amplicons of the correct size (Figure 1a) and sequence (data not shown) could be demonstrated in all three skin specimens. Real-time PCR quantitative analysis demonstrated that the expression of the various thyroid-specific genes was significantly lower than that observed in a pool of total RNA extracted from three normal human thyroid biopsies (Figure 1b), being 0.0020 ± 0.0006 ($P<0.01$) for TSH-R mRNA, 0.0069 ± 0.0001 ($P<0.01$) for TPO mRNA, 0.0016 ± 0.0010 ($P<0.01$) for Tg mRNA, and 0.0017 ± 0.0015 ($P<0.01$) for NIS mRNA.

The expression of the four genes was further analyzed in five primary cultures of normal human keratinocytes and dermal fibroblasts and compared with that observed in a normal strain of human thyrocytes in culture (HTU5). TSH-R transcripts were detected in all keratinocytes and dermal fibroblasts analyzed (Figure 1c), being 0.033 ± 0.013

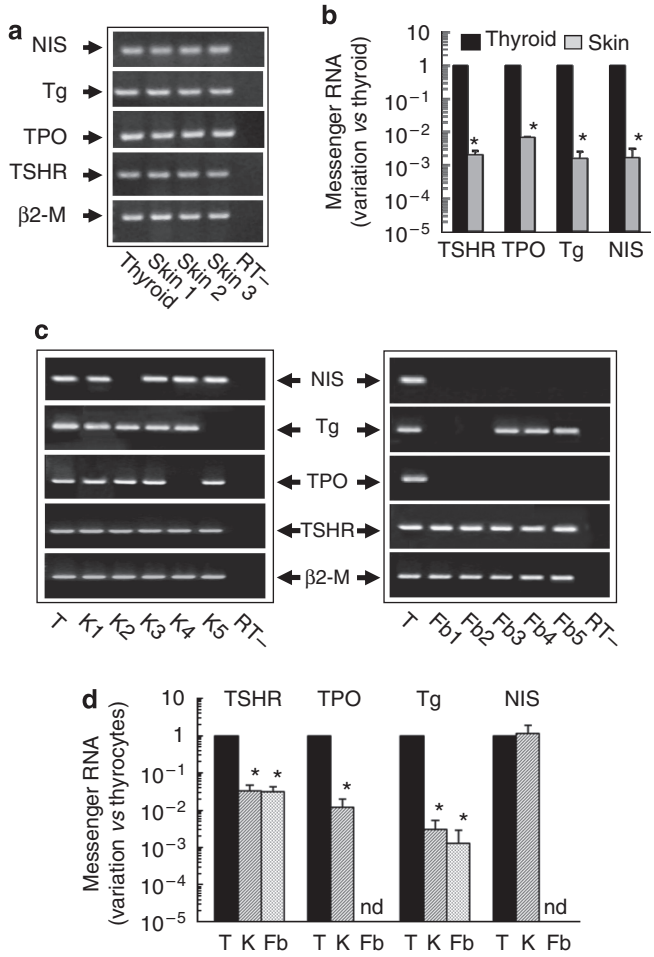

Figure 1. Quantitative RT-PCR analysis for NIS, Tg, TPO and TSH-R in skin and different strains of keratinocytes and fibroblasts. Panels (**a**) and (**c**) show representative agarose gel electrophoresis of the amplified products. Panels (**b**) and (**d**) depict the mRNA levels of the genes in whole human skin and in keratinocytes and fibroblasts compared with those in normal thyroid tissue or human thyrocytes, respectively. T, thyrocytes; K, keratinocytes; Fb, fibroblasts. *$P<0.01$.

($P<0.001$)-fold and 0.030 ± 0.011-fold, respectively, of that observed in human thyrocytes (Figure 1d). Specific NIS, Tg, and TPO mRNAs could also be detected in four of five strains of cultured keratinocytes. In particular, NIS mRNA levels did not significantly differ from those observed in human thyrocytes (1.13 ± 0.76), whereas those of TPO and Tg were, respectively, 0.012 ± 0.008 ($P<0.001$)- and 0.003 ± 0.002 ($P<0.001$)-fold the level detected in thyrocytes. On the other hand, NIS and TPO transcripts could not be detected in dermal fibroblasts, whereas Tg mRNA was expressed in three out of five primary cultures of fibroblasts, being 0.0013 ± 0.0015-fold of that present in thyrocytes (Figure 1c and d).

TSH-R protein in human skin samples from healthy individuals and cultured primary keratinocytes and fibroblasts

We next investigated the expression of the TSH-R at the protein level by western blot and immunohistochemistry in normal human skin biopsies, cultured keratinocytes, and dermal fibroblasts. The ability of the TSH-R antibody to specifically recognize the TSH-R protein was at first evaluated. To this end, western blot experiments were carried out on thyroid tissue extracts under reducing and nonreducing conditions. As expected, in reducing conditions, only one immunoreactive band of 53 kDa, corresponding to the α subunit of the TSH-R (Loosfelt *et al.*, 1992), was observed, whereas in nonreducing conditions, an immunoreactive band of approximately 120 kDa, corresponding to the holoreceptor, was seen (Supplementary Figure S1, panel A). Western blot conducted in reducing conditions, with extracts from thyroid tissue, human skin samples, and cultured keratinocytes and fibroblasts, showed an immunoreactive band of 53 kDa in all the analyzed tissues and cell type samples (Figure 2a). However, the filter exposure times required to visualize a band were longer for skin and cultured cells (20–30 min) with respect to thyroid tissue (1–2 min).

Immunohistochemistry experiments were carried out on both paraffin-embedded and frozen skin tissue sections from healthy donors. Thyroid tissue was also analyzed as control. As expected, TSH-R expression was confined to epithelial cells in the thyroid gland (Figure 2b). The immunoreactivity was predominantly cytoplasmic in paraffin-embedded sections, whereas it showed a basolateral expression pattern in most cells in cryosections (Supplementary Figure S1, panel B). Results obtained from skin specimens confirmed TSH-R expression in epidermis and dermal cells on both paraffin-embedded and frozen sections (Figure 2b and c). Keratinocyte staining was evident on all cell layers and appeared stronger in differentiated cells. To confirm that labeled cells in the dermis were fibroblasts, immunohistochemistry was carried out on serial frozen tissue sections using the TSH-R antibody and an antibody against prolyl-4-hydroxylase, a fibroblast-specific marker (Esterre *et al.*, 1992). The results showed that several of the TSH-R-positive cells in the dermis were also stained with the anti-prolyl 4-hydroxylase (Figure 2c). Double immunofluorescence with the same antibodies, both monoclonal, was also performed. Staining given by single immunofluorescence was comparable to that observed by immunohistochemistry for both antibodies; however, when the same section was treated with anti-TSH-R and anti-prolyl-4-hydroxylase, satisfactory results could not be obtained because of high background (data not shown).

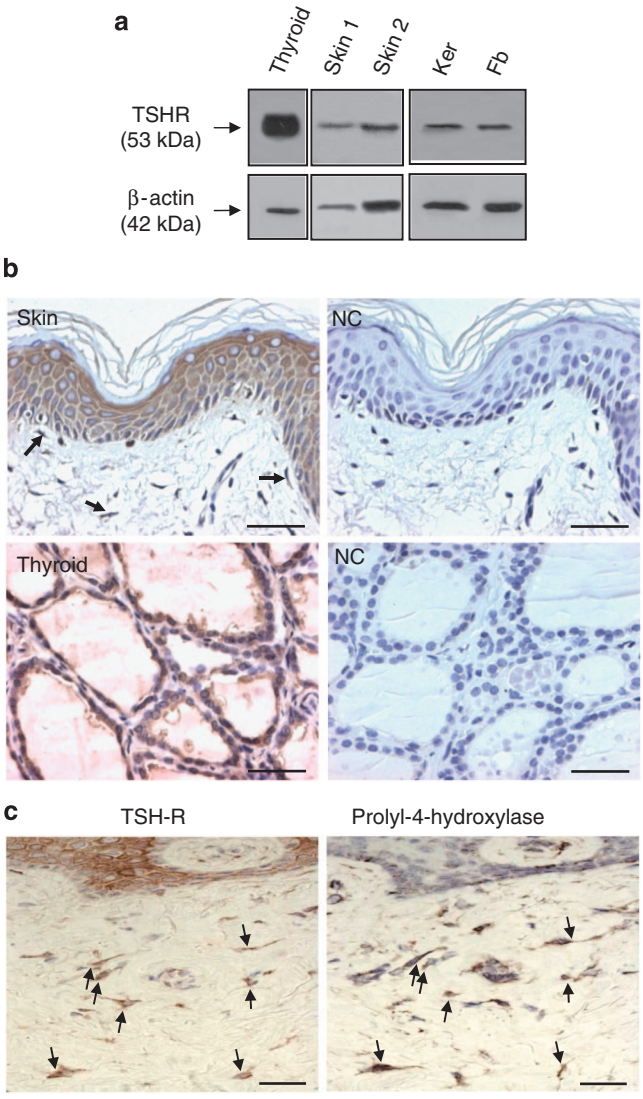

Figure 2. TSH receptor (TSH-R) expression in human skin and primary culture of keratinocytes and fibroblasts. (**a**) Western blot for detection of TSH-R α subunit in thyroid, skin, and cultured keratinocyte (Ker) and fibroblast (Fb) protein extracts. Note that the exposure times of the filters differed, being longer for skin tissue and cultured cells with respect to thyroid tissues. (**b**) Immunohistochemistry analysis of TSH-R on human thyroid tissue, used as positive control, and on healthy human skin sections. Immunoreactivity is evident in the follicular cells of the thyroid and in the epidermis and sparse cells in the dermis (arrows) in skin specimens. Negative controls (NC) were obtained by omitting the primary antibody. (**c**) Immunohistochemistry on serial frozen tissue sections using the TSH-R antibody and the antibody against prolyl 4-hydroxylase, a fibroblast marker. Arrows indicate dermal cells that are stained with both antibodies. Immunohistochemistry data reported are representative of one out of three normal human skin samples obtained from healthy donors. Bars = 30 μm.

Analysis of TSH-R activity in cultured keratinocytes and fibroblasts

We then analyzed keratinocyte and fibroblast proliferation following treatment of cultured cells with TSH. Because thyroid hormones have previously been shown to increase keratinocyte and fibroblast proliferation (Safer *et al.*, 2001), triiodothyronine (T_3) (10 nM, for 72 h) was used in these experiments as positive control. As reported in Figure 3a, T_3 treatment stimulated both keratinocyte and fibroblast BrdU incorporation 1.45 ± 0.05-fold ($P<0.01$) and 1.58 ± 0.03-fold, respectively ($P<0.01$). Similarly, TSH treatment (10 mUI ml^{-1} for 72 h) induced a significant increase in BrdU incorporation in both cell types 1.42 ± 0.05-fold ($P<0.01$) and 1.53 ± 0.06-fold, respectively ($P<0.01$). Treatment with both TSH (10 mUI ml^{-1}) and T_3 (10 nM) for 72 h did not result in additive or synergistic effects (data not shown).

The ability of TSH (10 mUI ml^{-1}, for 2 h) to cause intracellular cAMP accumulation in cultured keratinocytes and dermal fibroblasts was also investigated. The results demonstrated that TSH stimulates intracellular cAMP 1.59 ± 0.18-fold ($P<0.05$) in keratinocytes, but not in fibroblasts (Figure 3b).

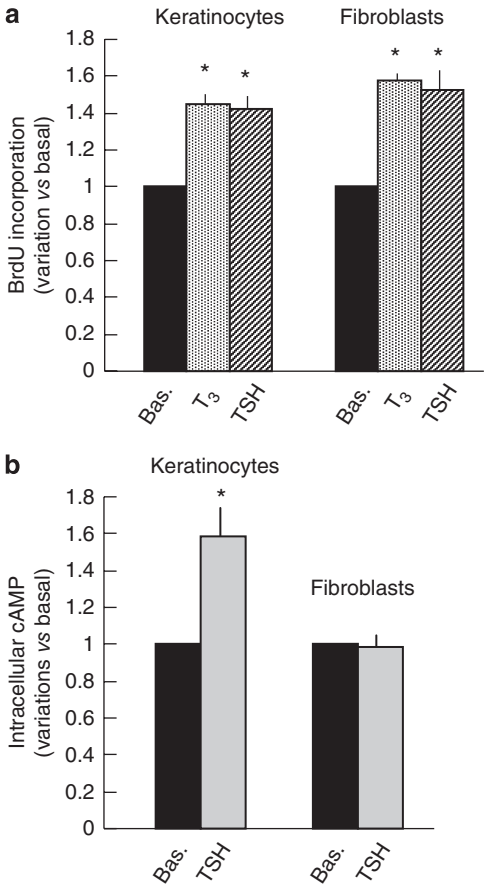

Figure 3. Effects of TSH and T_3 treatment on keratinocyte and fibroblast proliferation and cAMP accumulation. (**a**) To evaluate the effects of TSH (10 mUI ml^{-1}) and T_3 (10 mM) on keratinocyte and fibroblast proliferation, primary cultures were plated in 96 wells and treated for 72 h in the presence or absence of the two hormones. BrdU was added to the cells 16 h before the end of incubation time, and the incorporated BrdU was measured by ELISA. $^*P<0.01$. (**b**) Intracellular cAMP levels measured in basal and TSH-stimulated (10 mUI ml^{-1} for 2 h) keratinocytes and fibroblasts by ELISA. $^*P<0.05$.

Because TSH represents the main regulator of Tg, TPO, NIS, and TSH-R expression in thyrocytes, we analyzed its effect on the thyroid-specific gene expression in keratinocyte and fibroblast cell cultures. The results, reported in Supplementary Table S2, demonstrated the failure of TSH (10 mUI ml^{-1}, for 24 h) to affect Tg, TPO, NIS, and TSH-R mRNA levels in both cell types. Moreover, as assessed by a highly sensitive Tg luminescence immunoassay, no Tg protein in either cell supernatant could be detected in basal or TSH-stimulated (10 mUI ml^{-1}, for 24 h) conditions, whereas an increase of about 20-fold in Tg secretion was observed following TSH treatment of HTU5 cells used as positive control (data not shown).

Analysis of cultured keratinocyte functions after treatment with IgGs purified from serum of patients affected by autoimmune thyroid diseases

We next sought to verify whether IgGs purified from the serum of patients ($n=3$, see Table 1) affected by GD could affect BrdU incorporation and cAMP accumulation in primary cultures of human keratinocytes. As controls, cultured keratinocytes were treated with IgGs purified from serum of healthy individuals ($n=3$) or from patients affected by CLT ($n=4$), with a high level of circulating anti-Tg and/or anti-TPO autoantibodies (Table 1). As reported in Figure 4a, affinity-purified IgGs (1 mg ml^{-1}) from the serum of patients affected by GD, but not from patients with CLT, significantly stimulated proliferation with respect to IgGs purified from the serum of healthy individuals (1.48 ± 0.06 fold, $P<0.05$). In keeping with this finding, the analysis of cAMP accumulation indicated that only purified IgGs from patients affected by GD were able to induce a significant increase in intracellular cAMP (1.88 ± 0.20, $P<0.01$), compared with those from healthy controls (Figure 4b).

DISCUSSION

The evidence that thyroid dysfunctions often associate with skin alterations has been correlated with the capacity of thyroid hormones to directly affect skin cell activity by binding to their cognate nuclear receptors. The possibility that in AITDs circulating autoantibodies directed against specific thyroid molecules also contribute to skin dysfunctions has been put forward (Slominski *et al.*, 2002). Our results show that keratinocytes and dermal fibroblasts from biopsies of normal individuals express functional TSH-R. In addition, they provide evidence that circulating TSH-R autoantibodies may affect skin cell functions, supporting the concept that autoantibodies against thyroid-specific antigens may contribute to cutaneous symptoms in AITD.

To acquire insight into the possible role of thyroid-specific molecules in the skin, we first analyzed the expression of TSH-R, Tg, TPO, and NIS genes in normal human skin biopsies and primary keratinocyte and fibroblast cultures. A previous study by Slominski *et al.* (2002) described TSH-R mRNA expression in skin biopsies, in epidermal and hair follicle keratinocytes and melanocytes, in dermal and hair

Table 1. Female subjects selected for the analysis of the effect of purified IgG on keratinocyte functions

Age	TSH (mU l^{-1})	FT4 (pg ml^{-1})	Ab-Tg (U ml^{-1})	Ab-TPO (U ml^{-1})	Ab-TSH-R (UI l^{-1})	Diagnosis
33	2.13	12.3	<60	<60	<1	Healthy
33	1.81	14.8	<60	<60	<1	Healthy
49	1.83	12.9	<60	<60	<1	Healthy
55	6.79	10.5	2,507	4,514	<1	Chronic lymphocytic thyroiditis
66	15.21	12.1	1,310	11,690	<1	Chronic lymphocytic thyroiditis
36	1.62	11.2	430	1,380	<1	Chronic lymphocytic thyroiditis
38	2.39	10.1	3,855	3,238	<1	Chronic lymphocytic thyroiditis
38	<0.01	32.7	<60	195	11.76	Graves' disease
32	<0.01	24.6	107	631	5.46	Graves' disease
45	3.02	8.2	157	1,102	8.84	Graves' disease

Normal range values: TSH: 0.4–4 mU l^{-1}; FT4: 8–19 pg ml^{-1}; Ab-Tg: <60 U ml^{-1}; Ab-TPO: <60 U ml^{-1} Ab-TSH-R: <1 UI l^{-1}.

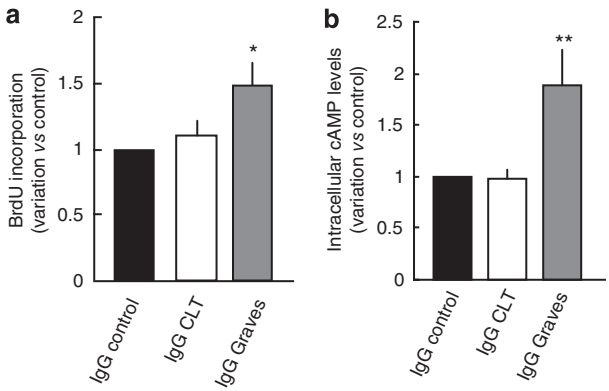

Figure 4. Proliferation and cAMP levels in human keratinocytes following treatment with IgGs. The effects of IgGs purified from serum of healthy females ($n=3$) or from that of female patients affected by chronic lymphocytic thyroiditis ($n=4$) or Graves' disease ($n=3$) on keratinocyte proliferation (**a**) or intracellular cAMP accumulation (**b**). *$P<0.05$, **$P<0.01$.

follicle fibroblasts, and in melanoma cell lines. Tg and NIS mRNAs, but not TPO mRNA, were also found to be expressed in the keratinocyte cell line HaCaT and melanoma cell lines. Our study confirms and further characterizes the expression of all four thyroid-specific genes in skin biopsies from healthy individuals and in a panel of primary keratinocyte and fibroblast cultures at low passage. However, Bodó *et al.* (2009) recently reported the expression of TSH-R and Tg in human hair follicles and that such expression was limited to the mesenchymal cells of the pilosebaceous unit, whereas it was not observed in interfollicular dermal fibroblasts and keratinocytes. Different skin locations (scalp versus buttock, shoulder, calf, or thigh analyzed here) and/or different reagents used (that is, anti-TSH-R antibodies) may give an explanation for such discrepant data. In the present study, of the four genes analyzed, only TSH-R mRNA was always found in all the primary cultures analyzed**.** To explain the diverse expression of the other genes in the cell strains analyzed, we looked at whether it could be related to differences in the body district or in the donor's age, but no correlation could be found. Alternatively, their differential expression could be ascribed to the different activities of the transcription factor(s) regulating the expression of the thyroid-specific genes in the analyzed primary cell cultures and skin cell types. In thyrocytes, the expression of thyroid-specific genes is sustained by the combined action of at least three transcription factors: thyroid transcription factor-1, forkhead/winged-helix domain transcription factor FoxE1, and the paired box 8 (Pax8) (Damante *et al.*, 2001). Even if the expression and function of these transcription factors in human skin remain to be fully investigated, the expression of thyroid transcription factor-1 in rat keratinocytes and in human hair follicle and that of FOXE1 in human epidermis have been described (Suzuki *et al.*, 1998; Eichberger *et al.*, 2004; Bodó *et al.*, 2009). Further investigation in this direction might help to clarify the significance of the differential expression of the Tg, NIS, and TPO genes in human skin.

The results of quantitative RT-PCR showed that, with the exception of NIS mRNA in keratinocytes, all the thyroid-specific gene mRNAs are present in the skin or in cultured keratinocytes and fibroblasts at levels much lower than those observed in, respectively, human thyroid tissues or thyrocytes in culture. This may suggest the presence of an illegitimate transcription of thyroid-specific genes in skin cells (Chelly *et al.*, 1989). However, we showed herein by immunohistochemistry and western blot experiments that the TSH-R gene, at least, is expressed at the protein level in skin biopsies and cultured keratinocytes and fibroblasts. This strongly argues against an illegitimate transcription and raises the question of the physiological role of TSH-R in skin functions. In this regard, a previous report demonstrated the ability of TSH-R to mediate TSH and anti-TSH-R antibody stimulation of cAMP in the immortalized human keratinocyte cell line HaCaT (Slominski *et al.*, 2002). In the present study, we could confirm these observations with normal keratinocytes and

demonstrated the ability of TSH to induce proliferation of keratinocytes and dermal fibroblasts, which, to our knowledge, has not previously been reported. Moreover, we showed that IgGs purified from the serum of patients affected by GD stimulate human keratinocyte proliferation. In keratinocytes, this effect is associated with the accumulation of intracellular cAMP, although we could not detect any cAMP increase after TSH treatment in dermal fibroblasts. The lack of a cAMP rise in fibroblasts may suggest the presence in this cell type of different intracellular signaling pathway(s) activated by TSH. In fact, TSH has been documented to induce, in addition to cAMP, the protein kinase C pathway in thyroid cells. Ellerhorst *et al.*, 2006 demonstrated the ability of TSH to stimulate the proliferation of two melanoma cell lines, WM793 and MeWo. In this study, TSH was shown to stimulate cAMP accumulation in WM793 cells, but not in MeWo cells, and to activate the MAPK pathway in both cell lines.

TSH represents the main regulator of thyroid-specific gene expression in thyrocytes. The finding of functional TSH-R in human keratinocytes and fibroblasts led us to investigate the effects of TSH treatment on keratinocyte and fibroblast Tg, TPO, NIS, and TSH-R mRNA levels. The results failed to show any effect of TSH on the expression of these genes. Similar observations were made by Sellitti *et al.* (2000a, b), who reported the expression of functional TSH-R and Tg in a primary culture of human kidney cells and the lack of effect of either TSH or cAMP analog on Tg expression. More recently, however, Bodó *et al.* (2009) reported the ability of TSH to induce extracellular cAMP accumulation and the Tg mRNA level in scalp hair follicles in organ culture (Bodó *et al.*, 2009).

Collectively, the above-reported experimental evidence indicates a role for TSH in human skin as already described in other extrathyroidal tissues in which the presence of functional TSH-R has been documented (Ümit Bağriaçik and Klein, 2000; Sellitti *et al.*, 2000a, b; Busuttil and Frauman, 2001; Slominski *et al.*, 2002; Ellerhorst *et al.*, 2006). The present data on the stimulation of proliferation of keratinocytes and dermal fibroblasts, along with those previously described on melanoma and kidney cells, suggest a mitogenic role for TSH in extrathyroidal target tissues (Sellitti *et al.*, 2000a; Ellerhorst *et al.*, 2006). This may have several implications, such as for the reported higher prevalence of hypothyroidism among melanoma patients with respect to the general population (Ellerhorst *et al.*, 2006). Moreover, our findings and those of Slominski *et al.* (2002) suggest that, in patients affected by GD, circulating anti-TSH-R autoantibodies may contribute, together with abnormal thyroid hormone levels, toward inducing skin alterations and also may make it reasonable to speculate that some forms of GD may result from autoimmune responses directed primarily against cutaneous TSH-R (Slominski *et al.*, 2002).

In this context, retrospective or prospective epidemiological studies aimed at evaluating the incidence of GD in patients who have a skin lesion (that is, skin inflammation, abnormal skin UV exposure, or medical exposure to radiation) may help to provide, although indirectly, evidence for the role of cutaneous TSH-R in the pathogenesis of GD. On the other hand, the ability of TSH to promote skin cell proliferation presents the possibility that this factor may have a role in enhancing cell response in those processes in which proliferation is required, such as wound healing.

In the human thyroid, TSH represents the main driver for both proliferation and differentiation of thyrocytes. The observation that TSH-R immunohistochemical staining is more intense in the epidermal upper layers suggests a possible role for the TSH/TSH-R axis in keratinocyte differentiation as well. However, whether TSH may affect, in addition to proliferation, keratinocyte and dermal fibroblast differentiation remains to be investigated.

With regard to the expression of the other nominally thyroid-specific genes, Tg, NIS, and TPO, their role in human skin, as in other extrathyroidal tissues, remains to be clarified (Sellitti *et al.*, 2000a; Sellitti *et al.*, 2000b; Ümit Bağriaçik and Klein, 2000; Busuttil and Frauman, 2001; Slominski *et al.*, 2002).

In conclusion, our data confirm the expression of thyroid-specific genes in human skin and show a mitogenic role for TSH and TSH-R autoantibodies in keratinocytes and fibroblasts. These findings suggest a role for TSH in the regulation of skin cell functions and indicate that circulating autoantibodies may contribute to skin alterations in the course of autoimmune thyroid diseases.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, Ham's F12 growth medium, F10 medium, L-glutamine, trypsin/EDTA, penicillin/streptomycin, Trizol reagent, oligo(dT)$_{12-18}$ primer 0.5 μg μl^{-1}, and M-MLV reverse transcriptase 200 U μl^{-1} were purchased from Invitrogen (Carlsbad, CA). BSA, trizma base, glycine, adenine, insulin, hydrocortisone, triiodothyronine, cholera toxin, and the polyclonal anti-actin antibody (AA20-33) were obtained from Sigma Chemical (St Louis, MO). Fetal bovine serum was purchased from HyClone (South Logan, UT). Precision Protein Standard, the Bradford protein assay kit, and electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). The Perfectprep Gel Cleanup Kit was obtained from Eppendorf (Hamburg, Germany). Microconcentrators Centricon 10 was from Millipore (Bedford, MA). Diaminobenzidine substrate system was from Dako Cytomation GmbH (Hamburg, Germany). The mouse monoclonal antibody raised against peptide 147–228 of the human TSH-R (Clone A9) and the mouse monoclonal antibody against prolyl 4-hydroxylase (5B5) were from GeneTex (San Antonio, TX). Anti-mouse and anti-rabbit peroxidase-conjugated antibodies and SuperSignals chemiluminescent substrate were from Pierce (Rockford, IL). Anti-mouse biotinylated and FITC- and TRITC-labeled antibodies and the Vectastain Elite ABC kit were from Vector Laboratories (Burlingame, CA). Protein A Sepharose CL-4B was obtained from Amersham Biosciences (Uppsala, Sweden). The BrdU cell proliferation kit and the FastStart DNA Master^{PLUS} SYBR Green I kit were from Roche Applied Sciences (Mannheim, Germany). The kit Parameter cAMP was from R&D System Europe (Abingdon, UK). Luminescence immune-assay kits for the determination of serum Tg, TSH, free T_4, and autoantibodies against Tg, TPO, and TSH-R were purchased from Brahms (Berlin, Germany).

Cell cultures and tissue samples

The normal strain of HTU5 was cultured as previously described (Curcio *et al.*, 1994). Of the five strains of normal human fibroblasts and keratinocytes, two were established from foreskin (samples 2 and 4), one from buttock (sample 1), one from shoulder (sample 3), and one from groin (sample 5) skin biopsies of five healthy individuals. Fibroblasts were grown in F10 medium supplemented with 10% fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin, and keratinocytes were cultured on a feeder layer of lethally irradiated 3T3-J2 murine fibroblasts (a gift from Howard Green, Harvard Medical School, Boston, MA) in Dulbecco's modified Eagle's medium and Ham's F12 (3:1 mixture) containing FCS (10%), insulin ($5\,\mu g\,ml^{-1}$), transferrin ($5\,\mu g\,ml^{-1}$), adenine (0.18 mM), hydrocortisone ($0.4\,\mu g\,ml^{-1}$), cholera toxin (0.1 nM), T_3 (2 nM), EGF ($10\,ng\,ml^{-1}$), glutamine (4 mM), and penicillin–streptomycin ($50\,IU\,ml^{-1}$), as previously described (Zambruno *et al.*, 1995). All the experiments on the primary cell cultures were carried out at cellular passages 2 to 4.

Paraffin-embedded skin tissue samples were obtained from surgical specimens of healthy female individuals confirmed as having normal thyroid function and not taking relevant medication at the time of harvest. One skin biopsy was from the buttock, one from the shoulder, and one from the calf. Frozen skin samples were from healthy female individuals undergoing plastic surgery (two biopsies were from the abdomen and one from the breast). Normal thyroid tissue fragments were obtained from patients subjected to thyroidectomy for benign follicular adenoma. This study was approved by the Ethics Committee of the Istituto Dermopatico dell'Immacolata. The Declaration of Helsinki protocols were followed, and patients gave their written informed consent.

Messenger RNA extraction and analysis

Tissue samples were homogenized in Trizol reagent, and total cellular RNA was extracted from tissue samples or cultured cells by the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Five micrograms of total RNA was reverse-transcribed, and cDNAs were used as a template for the subsequent quantitative PCR amplifications of the TSH-R, Tg, NIS, TPO, and β2-microglobulin as internal control, using specific primers (Supplementary Table S1). Controls for DNA contamination were obtained by omitting the reverse transcriptase or RNA during reverse transcription. Real-time PCR assay was carried out on a LightCycler instrument (Roche Diagnostics, Monza, Italy) using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics). Samples were processed in triplicate, and PCR-grade water was used as negative control. The PCR products were analyzed on a 2% agarose gel. To determine the specificities of amplified cDNAs, they were recovered from the gel and sequenced using the ABI Prism 377 DNA sequencer (PerkinElmer, Boston, MA). The crossing points (C_p) for each reaction were determined, and data were calculated using the $\Delta\Delta C_p$ method and the LightCycler Relative Quantification software, version 1.0 (Roche Diagnostics). The expression of thyroid-specific genes in keratinocytes and fibroblasts or in whole skin was normalized against the expression found in the normal strain of HTU5 or in normal human thyroid tissue, respectively, and reported as fold of variation.

Western blot

Normal skin and thyroid tissues were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 150 mM sodium chloride, 1 mM EDTA, 1 mM sodium fluoride, 1 mM AEBSF, $10\,\mu g\,ml^{-1}$ aprotinin, $10\,\mu g\,ml^{-1}$ leupeptin, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate in ddH_2O), sonicated, and centrifuged at 10,000 r.p.m. for 10 min. The supernatants were then recovered, and protein concentrations were determined by the Bradford assay. Fifty micrograms of extracts was supplemented with Laemmli buffer (120 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) with or without β-mercaptoethanol (1%), electrophoresed on a 12.5% polyacrylamide gel, and transferred onto nitrocellulose membranes using the Bio-Rad Mini Trans-Blot Cell system. The membranes were then washed with TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and saturated with 5% low-fat milk in TBST for 2 h at room temperature. Incubations with primary antibodies against the TSH-R (1:5,000) or actin (1:1,000) were carried out in TBST containing 2% low-fat milk at 4 °C overnight. The membranes were then washed and incubated with anti-mouse (1:1,000) horseradish peroxidase-conjugated secondary antibodies in TBST plus 2% low-fat milk for 2 h at room temperature. After several washes with TBST, western blots were revealed using the chemiluminescence Super Signal kit.

BrdU incorporation assay

Keratinocytes and fibroblasts were plated in 96-well plates at a density of 2×10^4 cells per well in a medium without serum, EGF, T_3, and cholera toxin and in the presence of 0.1% bovine serum albumin (BSA). On the following day, the cells were washed and incubated in triplicate with TSH ($10\,mUI\,ml^{-1}$), T_3 (10 nM), or medium alone (control) for 72 h. In separate experiments, the cells were incubated for 72 h with immunoglobulins purified from the blood serum of control subjects or from patients affected by CLT or GD. BrdU was added to the cells 16 h before the end of the incubation time, and its incorporation was analyzed using the cell proliferation kit according to the manufacturer's instructions.

cAMP assay

Keratinocytes and fibroblasts were cultured in 96-well plates for 4 days in medium without serum, EGF, T_3, and cholera toxin and in the presence of 0.1% BSA. The cells were then washed and incubated in triplicate with TSH ($10\,mUI\,ml^{-1}$) or medium alone (control) for 2 h. In different experiments, the cells were incubated for 2 h with immunoglobulins purified from the blood serum of control subjects or patients affected by CLT or GD. At the end of the incubation time, intracellular cAMP levels were measured with the Parameter cAMP kit according to the manufacturer's instructions.

Thyroglobulin assay

Normal human keratinocytes and dermal fibroblasts were cultured for 4 days in medium without serum, EGF, T_3, or cholera toxin and in the presence of 0.1% BSA. Cells were then cultured in the absence or presence of TSH ($10\,mUI\,ml^{-1}$) for 24 h. At the end of the incubation, the conditioned media were collected and Tg was measured by a highly sensitive luminescence immune assay as previously described (Morgenthaler *et al.*, 2002). Conditioned media from normal human thyrocytes cultured in the absence or presence of TSH ($10\,mUI\,ml^{-1}$) were used as control.

Immunohistochemical analysis

Four-micrometer-thick paraformaldehyde-fixed and paraffin-embedded sections of normal human thyroid and skin were deparaffinized, rehydrated, and treated for 20 min with 0.3% H_2O_2 in phosphate-buffered saline (PBS). Sections were preincubated with 3% BSA in PBS for 1 h at room temperature and then incubated with the primary antibody raised against human TSH-R (1:500) overnight at 4 °C. The sections were then washed and incubated with the secondary anti-mouse biotinylated antibody (1:150) in PBS for 1 h at room temperature, washed again, and incubated for 1 h with an avidin–biotin–peroxidase complex (Vectastain Elite ABC kit). For the analysis of frozen tissues, 3-μm-thick sections from thyroid and skin specimens were used, and samples were fixed for 10 min in cold acetone. For TSH-R expression analysis, the method described above was followed using the primary antibody diluted 1:250. Prolyl 4-hydroxylase was detected by incubating with the primary antibody (1:50) for 1 h at room temperature, followed by incubation with the biotinylated anti-mouse antibody (1:200) for 30 min at room temperature. Immunoreactivity was visualized by a peroxidase reaction using diaminobenzidine tetrahydrochloride in H_2O_2 and counterstaining with hematoxylin. For immunofluorescence analysis, an FITC-conjugated secondary antibody was used to detect TSH-R and a TRITC-conjugated one for prolyl 4-hydroxylase. When used together on the same specimen, the primary antibodies were preincubated with an excess (molar ratio) of the secondary antibody, and the complexes were simultaneously added to the tissue sample. Negative controls were obtained by omitting the primary antibody or by incubating specimen sections with nonspecific IgGs instead of with primary antibody.

Purification of immunoglobulins from blood serum

Blood sera were obtained from three female patients affected by GD, from three female patients affected by CLT, and from four healthy females enrolled at the Thyroid Clinic of the Policlinico Umberto I of Rome. All patients gave their informed consent. Serum determination of TSH, free T_4 (FT_4), and autoantibodies against Tg, TPO, and TSH-R was carried out using commercially available kits according to the manufacturer's instructions. IgGs were purified from 0.5 ml of blood serum samples by affinity chromatography using Protein A Sepharose CL-4B columns in accordance with the manufacturer's instructions (Miller and Stone, 1978). Briefly, 0.5 ml of serum was diluted in PBS and loaded on a column containing approximately 100 mg of PBS-equilibrated Protein A Sepharose. After an overnight incubation at 4 °C, unbound material was eluted by three washes with 5 ml of PBS. Bound IgGs were eluted with 2.5 ml of glycine chloride 100 mM (pH 2.7), collecting 0.5 ml fractions. The collected IgG fractions were immediately neutralized (pH 7.0) with Tris-base 1 M. The protein concentration of each fraction was estimated using the Bradford assay and concentrated to 10 mg ml^{-1} using Microconcentrators Centricon 10. To verify IgG purification, a small aliquot of each sample was run on 10% SDS–PAGE. After Coomassie blue staining, only two bands at about 50 and 30 kDa, corresponding to the molecular weights of the Ig heavy and light chains, respectively, were observed (data not shown).

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance of differences was determined using the mean values of replicates of three independent experiments, each performed at least in triplicate and evaluated by the two-tailed Student *t*-test. The differences were considered to be statistically significant if the *P*-values were lower than 0.05.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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