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Protective effects of taurine on human hair follicle grown *in vitro*[1]

C. Collin*, B. Gautier*, O. Gaillard*, P. Hallegot†, S. Chabane*, P. Bastien†, M. Peyron†, M. Bouleau†, S. Thibaut*, F. Pruche*, A. Duranton* and B. A. Bernard*
L'Oréal Recherche, *Clichy et †Aulnay-sous-bois, France

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Synopsis

Taurine is a naturally occurring β-amino acid produced by methionine and cysteine metabolism. It is involved in a variety of physiological functions, including immunomodulatory and antifibrotic. Taking advantage of the ability of human hair follicle grown *in vitro* to recapitulate most of the characteristic features of normal hair follicle *in vivo*, we studied (i) taurine uptake by isolated human hair follicles; (ii) its effects on hair growth and survival rate; and (iii) its protective potential against transforming growth factor (TGF)-β1, an inhibitor of *in vitro* hair growth and a master switch of fibrotic program. We showed that taurine was taken up by the connective tissue sheath, proximal outer root sheath and hair bulb, promoted hair survival *in vitro* and prevented TGF-β1-induced deleterious effects on hair follicle.

Résumé

La taurine est un β-amino acide naturel qui dérive du métabolisme de la méthionine et de la cystéine. Elle est impliquée dans un grand nombre de fonctions physiologiques, et présente en particulier des activités immuno-modulatrices et antifibrotiques. En s'appuyant sur le modèle du follicule pileux *in vitro*, qui reproduit de nombreuses caractéristiques du follicule normal *in vivo*, nous avons étudié (i) l'incorporation et la distribution de la taurine dans un follicule pileux isolé, (ii) son effet sur la croissance et la survie du follicule et (iii) son rôle protecteur vis-à-vis du TGF-β1, à la fois inhibiteur de la croissance du follicule et inducteur du programme fibrotique. Nous montrons que la taurine est captée par la gaine conjonctive, la gaine externe proximale et le bulbe, assure la survie du follicule *in vitro* et s'oppose à l'effet délétère du TGF-β1 sur le follicule pileux.

Correspondence: Bruno A. Bernard, L'Oréal Centre C.Zviak, 90 rue du Général Roguet, 92110 Clichy, France. Tel.: +33 (0)1 47567599; fax: +33 (0)1 47567078; e-mail: bbernard@rd.loreal.com

Introduction

Taurine (2-aminoethanesulphonic acid) is a naturally occurring β-amino acid produced by methionine and cysteine metabolism, which differs from other amino acids by having a sulphonic acid (SO_3H) group instead of carboxylic acid (COOH). In humans, taurine is frequently the free amino acid found at the highest concentration in numerous cell types, but because mammalian ability to synthesize taurine is limited [1,2], dietary intake plays an important role in maintaining body taurine pool [3]. Conserved through evolution from the most primitive life forms, taurine is involved in a variety of physiological functions [2,4–6], including conjugation of bile acids, regulation of calcium homeostasis, membrane stabilization, antioxidation, detoxification, neuromodulation, retinal and cardiac function, brain development and cellu-

lar osmoregulation. Last but not least, taurine is endowed with immunomodulatory [3,7] and antifibrotic properties [8–10].

Although taurine is primarily synthesized in the liver and brain, high levels have been found in several tissues including the heart, retina and gut [2]. In rat skin, taurine was essentially found in the epidermis and outer root sheath (ORS) near the hair follicle ostium [11]. The ORS just below the sebaceous gland duct and the peripheral part of the sebaceous gland was also reported to have a high taurine content [11]. The epidermal distribution of taurine was further confirmed by immunohistochemistry in the keratinocytes of granular and upper spinous layers of both dog and rat epidermis [12]. In human epidermis, the taurine transporter (TAUT) was immunologically detected in granular and spinous layers, taurine behaving as a key osmolyte required for proper keratinocyte hydration and protection from both osmotically and UV-induced apoptosis [13]. This epidermal carrier was functionally characterized as a sodium- and chloride-dependent high-affinity taurine transporter [13,14], very similar to the transport system described in liver, placenta, intestine, retina, kidney, choroids plexus and blood–brain barrier.

If taurine distribution and functions in human epidermis begin to be understood, it remains that nothing is known about taurine function in human scalp hair follicle. Yet, the hair follicle is an epidermal appendage the miniaturization of which is often associated with perifollicular lymphocytic infiltrates and fibrotic processes [15–17], which are acknowledged targets of taurine. Taking advantage of the ability of human hair follicle grown *in vitro* to recapitulate most of the characteristic features of a normal hair follicle, e.g. keeping producing hair at a rate of 0.3 mm day^{-1} with a proper keratin synthesis and organization [18], we decided to study (i) taurine uptake by isolated human hair follicles; (ii) its effect on hair growth and survival rate and (iii) its protective potential against transforming growth factor (TGF)-β1, an inhibitor of *in vitro* hair growth [19] and a master switch of fibrotic program [20,21].

Materials and methods

Materials

Taurine and recombinant TGF-β1 were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). [1,2-^{14}C] taurine (119.2 mCi $mmol^{-1}$) was obtained from Perkin Elmer (Courtaboeuf, France). [1,2-$^{13}C_2$] taurine was obtained from Euriso-Top (St Aubin, France).

Taurine detection in plucked hair

Outer root sheaths were dissected from 20 plucked anagen hair follicles and homogenized in 100 μL of sterile water. A volume of 60-μL sample was mixed with 60 μL of 50 mM lithium citrate buffer (pH 2) and kept frozen at −20°C until high-performance liquid chromatography (HPLC) analysis. At this pH, all amino acids are positively charged and can be separated by cation-exchange chromatography. For amino acid determination, 80 μL of aqueous extract was loaded on an amino acid analyser Hitachi L-8500 system as previously described [22]. The structural determination of taurine in hair follicle extracts was carried out using mass spectrascopy (HPLC/MS) and tandem mass spectroscopy (MSMS) with electrospray ionization in the negative-ion mode on a Finnigan LTQ linear ion-trap mass spectrometer equipped with a Surveyor quaternary pump (Thermo Electron, SA, Courtaboeuf, France). Tandem mass spectrometry was recorded in the negative-ion mode with a collision energy of 63% as expressed with Excalibur 1.4 version (Thermo Finnigan, Warrington, UK). A 10 μL of three different aqueous extracts of hair follicles was directly injected onto a 250 mm × 4.6 mm Atlantis C18 column packed with 5-μm particles. Taurine was eluted at 2.9 min when using a 0.1% formic acid aqueous mobile phase and was identified by comparing its retention time and MSMS spectrum with those of a reference sample (Fluka 86239 batch 436337/1 52203056).

Isolation of human hair follicles and *in vitro* culture

Human scalp biopsies were obtained from facelift surgery after informed consent of healthy volunteers. Individual human terminal scalp hair follicles were obtained and isolated as previously described [18]. The hypodermis was separated from dermis using a scalpel blade and hair follicles were then isolated from the subcutaneous fat with fine forceps, under a stereo dissecting microscope. Hair follicles were incubated at 37°C in a water-saturated atmosphere of 5% CO_2/95% air and cultured for 10 days in 24-well cell cul-

ture cluster containing 500 μL of William's E medium (Invitrogen SARL, Cergy Pontoise, France), supplemented with L-glutamine (2 mM), insulin (10 μg mL^{-1}), hydrocortisone (40 ng mL^{-1}) and antibiotics (1%) [23]. Follicles were re-fed three times a week. In separate experiments, hair follicles were cultivated in William's E medium containing 1 ng mL^{-1} TGF-β1 in the presence or absence of 1 mM taurine. Hair growth (elongation in mm) was assessed on a daily basis, using an inverted microscope (Leica, Rueil Malmaison, France) fitted with an eyepiece graticule.

In vitro uptake of taurine

Freshly dissected individual hair follicles were incubated in William's E medium (see above) for 24 h at 37°C in the presence of [1,2-^{14}C] taurine at 1.0 μCi 500 μL^{-1} or 3 days in the presence of [1,2-$^{13}C_2$] taurine 1 mM. [1,2-^{14}C] taurine medium was then aspirated, and replaced by growth medium containing 1 mM unlabelled taurine for a further 24-h incubation. Follicles were then harvested, washed in Williams E medium and embedded in Tissue-Tek OCT compound (Miles, Naperville, IL, U.S.A.), frozen in a mixture of dry-ice/EtOH and stored at −80°C. In the [1,2-$^{13}C_2$] taurine experiment, samples were fast-frozen in liquid nitrogen prior to be stored.

Preparation of cryo-processed samples

Hair follicles were fast-frozen to liquid nitrogen temperature, then lyophilized and embedded in epoxy resin. With a diamond knife, longitudinal sections of follicles were prepared for *in situ* high-resolution mass spectrometry detection of ^{13}C using a NanoSIMS (Cameca, Courbevoie, France).

Preparation of cryosections

As previously described for full-thickness scalp biopsies [24] and hair follicles [18], longitudinal frozen sections (7 μm) were prepared on a cryostat CM3050 (Leica) whose chamber temperature was set at −35°C. Sections were then air-dried and stored at 4°C. For histological observation, some sections were stained in haematoxylin–eosin (H&E). For direct visualization and analysis of [1,2-^{14}C] taurine uptake into hair follicles, longitudinal sections (7 μm) were observed using a M25 μ-imager® from Biospace Mesures Inc. (Paris, France) as previously described [25].

Immunolabelling

Indirect immunolabelling was carried out essentially as described previously [24], using primary monoclonal antibodies against α-smooth muscle actin (α-SMA, clone asm1, Cymbus, dilution 1/10) or rabbit anti-TAUT antiserum (Alpha Diagnostics, San Antonio, TX, USA; dilution 1/30). Secondary antibodies fluorescein isothiocyanate-conjugated goat antimouse (dilution 1/100; Jackson Laboratories, West Baltimore, PA, USA) or biotinylated goat antirabbit (dilution 1/100; Dako, Trappes, France) were used. Nuclei were revealed by diamidino-2-phenylindole (DAPI) staining. Immunolabelling was analysed using a Zeiss Axioscop microscope (Carl Zeiss, Oberkochen, Germany).

Statistics

Growth curves for control and 1 mM taurine groups were displayed as the mean elongation as a function of time. The effect of 1 mM taurine on hair growth was tested using a mixed model of variance with follicles taken as random effect and time, treatment group, and their interaction as fixed effects [26]. The effect of treatment was based on the statistical significance of the interaction term using Fisher's test.

Survival curves for the control and 1 mM taurine groups were estimated using Kaplan–Meier survival curves [27] and compared using the Breslow's test [28] with the follicles still alive at day 13 considered in the survival analysis as right censored. Due to the presence of numerous missing data, which could bias the global growth analysis, an endpoint analysis was also carried out by considering the final growth of each follicle. The distribution by treatment groups of hair follicle global growth was displayed using a box-plot nonparametric graphical representation, which represented the median and the interval between the 25th and 75th percentiles. Between group comparison was tested using a corresponding exact Wilcoxon rank test procedure. This analysis could also be viewed as a sensitivity analysis. The significance level for the statistical tests was been fixed at 5%. The analysis was carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and SAS 8.2 (SAS Grégy-Sur-Yerres, France) statistical software.

Results

Free taurine is present in human hair follicle ORS

As shown on a representative amino acid chromatogram of ORS aqueous extract (Fig. 1a), a peak of taurine was detected at a retention time of 2.73 min on the Hitachi L-8500 amino-acid analyser. Similar results were obtained from two distinct donors, the estimated amount of taurine being in the range of 0.1 μg/individual ORS. The presence of taurine in these aqueous samples was further confirmed by LC/MS/MS, on three independent 10-μL samples. Only one peak was eluted at 2.9 min from the Atlantis C18 column (Waters, St-Quentin-en-Yvelines, France), when using a 0.1% formic acid aqueous mobile phase. It was identified as taurine by comparing its retention

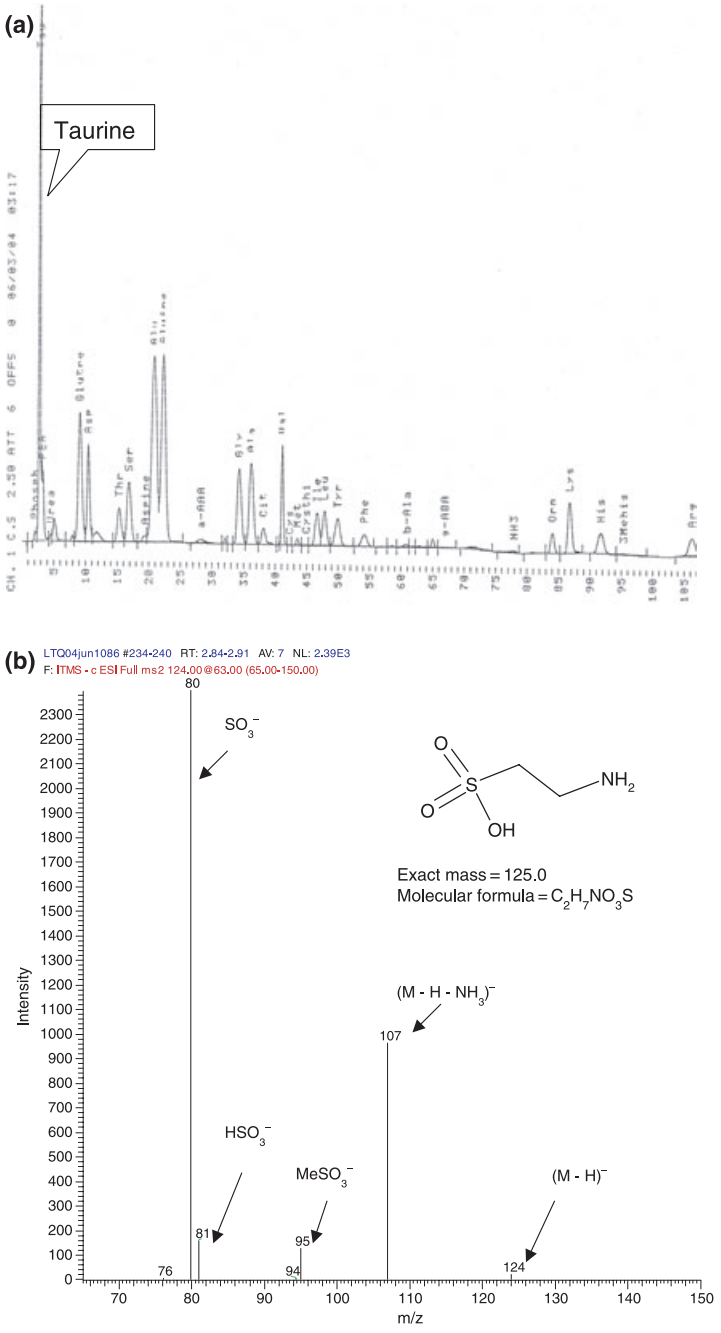

Figure 1 (a) Representative high-performance liquid chromatography chromatogram of free amino acids extracted from plucked hair follicle outer root sheath. Note the peak of taurine at a 2.73 min retention time. (b) Product-ion MSMS spectrum of the quasi molecular ion $[M-H]^-$ of taurine present in an aqueous hair follicle extract.

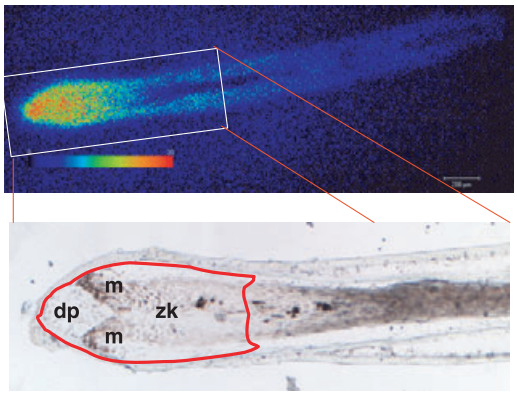

Figure 2 Distribution of [1,2-^{14}C] taurine in isolated hair follicle after a 24-h incubation followed a 24 h chase period. Note the strong taurine uptake in the hair bulb (dp, dermal papilla; m, matrix; kz, keratogenous zone).

time and MS/MS spectrum with those of a reference sample (Fluka 86239 batch 436337/1 52203056). The main fragments and their relative intensities observed on the ESI$^-$/MS/MS spectrum of taurine in three extracts were identical to those of the reference sample ($m/z = 124$ [M-H]$^-$, $m/z = 107$ [M-H-NH$_3$]$^-$, $m/z = 95$ $CH_3SO_3^-$, $m/z = 81$ HSO_3^- and $m/z = 80$ SO_3^-, base peak of the spectra) (Fig. 1b).

Taurine is taken up by human hair follicle *in vitro*

As taurine was readily detectable in isolated ORS from plucked hair, we then addressed the question of taurine uptake by intact hair follicle, considering the limited ability of mammals to synthesize taurine and the requirement of dietary intake in maintaining body taurine pool [1,2]. Freshly dissected human hair follicles were thus incubated for 24 h in the presence of [1,2-^{14}C] taurine. After a further 24 h chase period in the presence of unlabelled taurine, follicles were embedded in Tissue-Tek OCT compound, frozen on dry ice/ethanol, and processed for direct radioactivity detection by digital autoradiography on a M25 μ-imager® from Biospace Mesures Inc. [25]. Taurine uptake was evidenced in both ORS and hair bulb, a region of intense metabolic activity, cell division and hair shaft synthesis (Fig. 2). Due to the 10 μm resolution of digital autoradiography, we further studied taurine distribution by the more resolutive NanoSIMS technology. After incubation in the presence of [1,2-$^{13}C_2$] taurine, *in situ* detection of ^{13}C by NanoSIMS unambiguously revealed the presence of labelled taurine in the dermal sheath in the hair bulb zone, in the cytoplasm of ORS cells (Fig. 3) and matrix cells (data not shown). Whether taurine reached dermal papilla remained an open question.

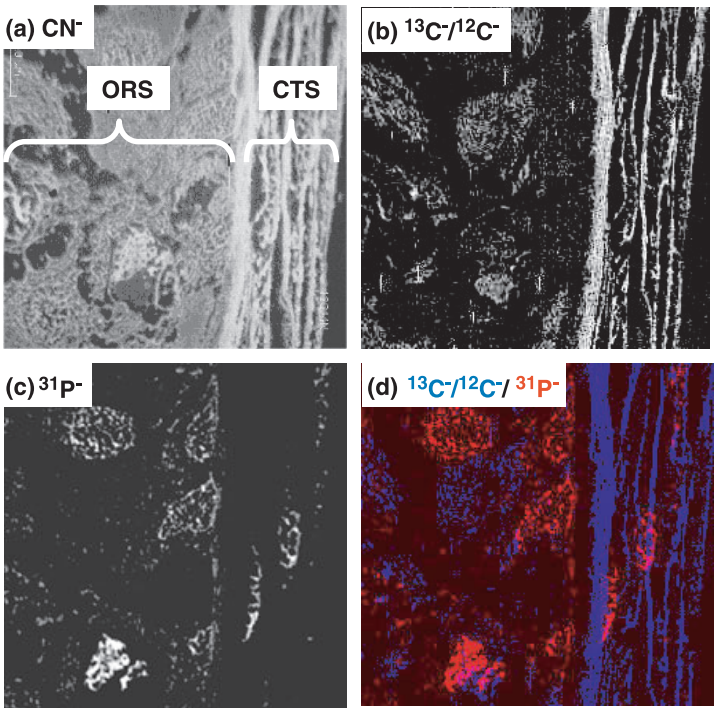

Figure 3 (a) Distribution of CN$^-$ ions, originated from proteins and revealing the histology of a 40 × 40μm area in the hair bulb. We can identify, from the right to the left, the dermal sheath and the outer root sheath (ORS); (b) distribution of labelled taurine, revealed by the $^{13}C^-/^{12}C^-$ map. Bright dots represent pixels with a value above the terrestrial value of 1% due to an accumulation of labelled taurine; (c) $^{31}P^-$ distribution map (mainly DNA) where nuclei can be localized; (d) colour-coded superimposed $^{13}C^-/^{12}C^-$ (in blue) and $^{31}P^-$ (in red) maps precisely identifying cytoplasm as the structure where labelled taurine accumulated in ORS cells.

To further confirm taurine uptake by human hair follicle, we immunolocalized the taurine transporter TAUT on full-thickness scalp biopsies (Fig. 4). As previously observed [13], TAUT transporter was detected throughout epidermis up to the stratum granulosum, papillary- and reticular dermis being negative. With respect to human hair follicle, TAUT transporter was indeed detected in the proximal ORS, the innerroot sheath (IRS) (mainly Henle's layer) and to a lesser extent in hair matrix and keratogenous zone. Interestingly, both dermal sheath- and dermal papilla fibroblasts were also labelled. TAUT expression was thus in agreement with both [1,2-^{14}C]- and [1,2-$^{13}C_2$]-taurine distribution in hair follicle.

Taurine prolongs the survival rate of human hair follicles *in vitro*

Considering the vast array of physiological functions taurine is endowed with and the fact that isolated hair follicles can pick it up as shown above, we next questioned whether taurine could influence hair growth and survival *in vitro*.

The highly significant treatment × time effect in the repeated measurement analysis (Fisher test, $P = 0.0087$) demonstrated a statistically significant higher hair elongation in the presence of 1 mM taurine with respect to control (Fig. 5a). This was confirmed by endpoint box-plot analysis, based on the global growth, with a *P*-value just below the 5% significance level in between groups comparison (exact Wilcoxson rank test, $P = 0.058$). The quartiles for the global growth were higher in the 1 mM taurine group than control (Fig. 5b).

Moreover, the survival probability was significantly improved in the presence of 1 mM taurine (Breslow test, $P = 0.0465$), as shown by the Kaplan–Meier survival curves (Fig. 5c).

Taurine blocks deleterious effect of TGF-β1 on human hair follicle

It is known that hair growth *in vitro* can be modulated by many factors. For instance cyclosporin A

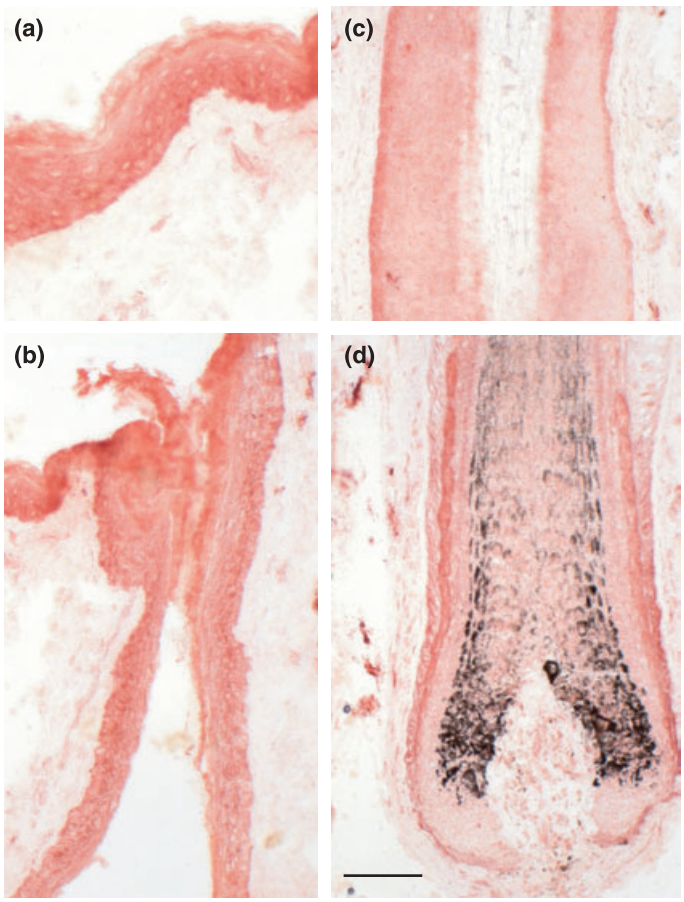

Figure 4 Immunohistochemical localization of TAUT in scalp epidermis and longitudinal frozen section of human hair follicle. (a) epidermis, (b) infundibulum, (c) mid-outer root sheath and (d) bulb. Bar = 25 μm.

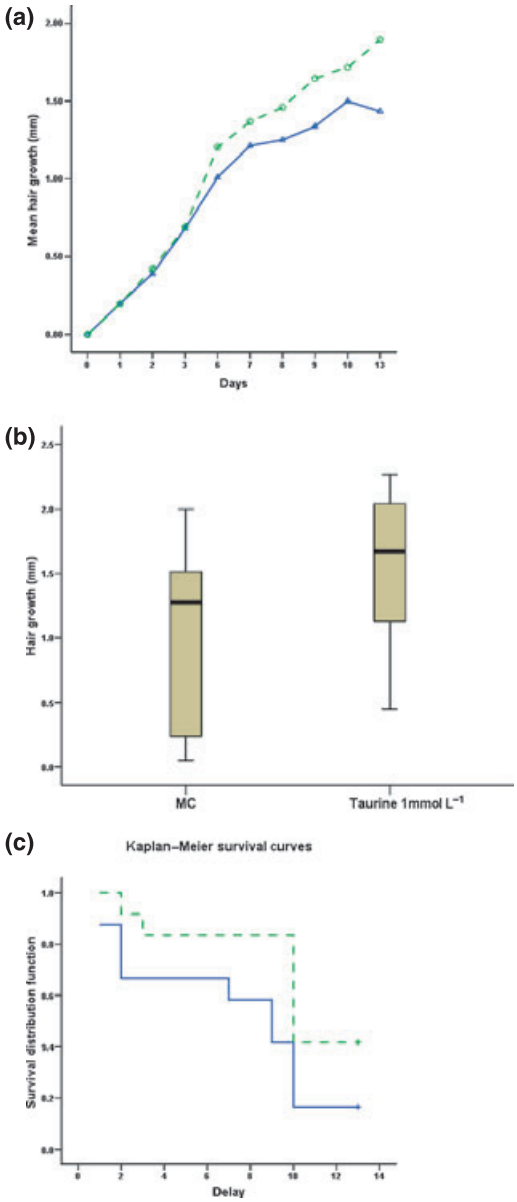

Figure 5 (a) *In vitro* hair growth as a function of time in the absence (—) or presence (- - -) of 1 mM taurine; (b) end-point box-plot of *in vitro* hair growth for absence vs. presence of 1 mM taurine. (c) Kaplan–Meier survival curves of hair follicles grown *in vitro*, in the absence (—) or presence (- - -) of 1 mM taurine. Note that survival is significantly improved in the presence of 1 mM taurine *in vitro* ($P = 0.0465$).

[29], IGF-1 [30] and glucose [31] support *in vitro* hair growth. On the opposite, interleukin-1α [32] and TGF-β1 [19] inhibit *in vitro* hair growth. Moreover, TGF-β1 is considered as a master switch of fibrotic program [21]. Since early features of androgenetic alopecia include perifollicular infiltrates and fibrosis [15–17] and since taurine has been reported to have immuno-modulatory [3,7] and antifibrotic properties [8–10], we wondered whether taurine not only could maintain hair growth and survival *in vitro* (see above) but also prevent deleterious effects of TGF-β1 on hair follicle as well. Indeed, when hair follicles were grown *in vitro* for 6 days in the presence of 1 ng mL^{-1} TGF-β1, a characteristic and reproducible curvature of the follicles was observed at the level of the bulb while no such bending was noticed when follicles were co-incubated with 1 mM taurine. In a representative experiment (Fig. 6), the curvature of the bulb was noticed in eight of nine TGF-β1-treated follicles, while this effect was prevented in nine of nine taurine-treated follicles. In response to TGF-β1, α-sma labelling and DAPI staining evidenced the presence of hyperplastic fibroblasts in contracted and thickened connective tissue sheath, which was the indicative of early fibrotic events (Fig. 7a,b). Conspicuously, 1 mM taurine opposed these effects (Fig. 7b,d): it prevented both connective tissue sheath thickening and hair bulb bending.

Discussion

Up to now, taurine had been detected in rat, dog [11,12] and human epidermis together with its TAUT transporter [13], but its functional roles in keratinocytes remained unknown. It could have a physiological role in cysteine metabolism, a key amino acid in keratin synthesis and/or be involved in the modulation of calcium levels [33], a key factor that controls terminal differentiation in keratinocytes. Nothing was reported with respect to the hair follicle except that taurine was found in the ORSs near the opening of hair follicles in mouse skin [11]. Here, we showed that taurine was one of the most abundant free amino acids of the human hair follicle ORS. By digital autoradiography, we found that [1,2-^{14}C] taurine was picked up by both proximal ORS and bulb area of isolated human hair follicle. One could not exclude that labelled taurine could also accumulate in other hair follicle compartments such as IRS, matrix, dermal sheath and dermal papilla, as the presence of TAUT taurine transporter was immuno-detected in ORS, IRS, dermal papilla and to a lesser extent in keratogenous zone, matrix and dermal sheath.

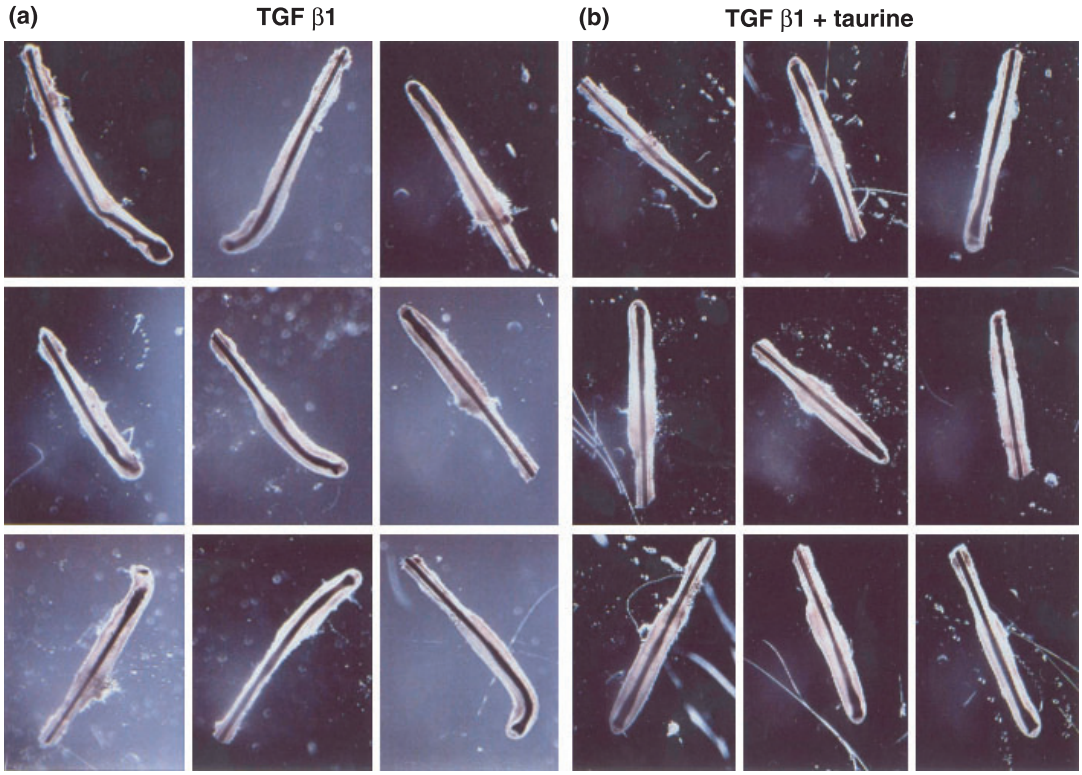

Figure 6 Gross morphology of human hair follicles grown *in vitro* for 6 days in William's E medium containing 1 ng mL^{-1} transforming growth factor (TGF)-β1, in the absence (a) or presence (b) of 1 mM taurine. Note the characteristic curvature of the hair bulb observed in response to TGF-β1 treatment and the preventing effect of taurine.

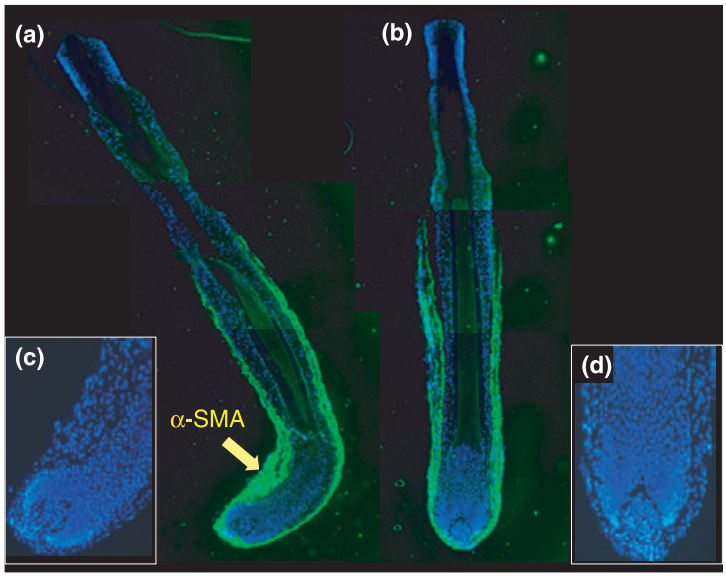

Figure 7 Effect of transforming growth factor-β1 treatment on hair follicle connective tissue sheath, in the absence (a, c) or presence of 1 mM taurine (b, d). (a, b) α-sma staining; (c, d) diamidino-2-phenylindole staining.

The resolution of the μ-imager was however not sharp enough to definitely ascertain such distribution. We thus decided to apply NanoSIMS technology, recently successfully adapted to human hair research [34]. The high spatial resolution of the instrument, its high sensitivity and its capability

for parallel detection [35] indeed permit the investigation of metabolisms of suitably labelled molecules. Minute accumulation of any isotope, used as a label, above its terrestrial value, can be detected in organelles. This technical breakthrough allowed the detection of [1,2-$^{13}C_2$] taurine at a spatial resolution two orders of magnitude better than attainable by digital autoradiography. We precisely identify cytoplasm as the accumulation site of taurine in proximal ORS cell and dermal sheath fibroblasts.

The site of taurine uptake, i.e. the hair bulb, is an area where a highly active cellular proliferation takes place, one of the most active in the whole body and from which all different follicular compartments originate [36]. This compartment is structurally and functionally surprisingly stable, since, when grown in a totally defined medium, isolated hair follicle keeps producing a normal hair fibre [18,37]. Moreover, survival rate *in vitro* is increased when isolated human hair follicles are grown in the presence of taurine. These findings strongly suggest that taurine could be involved in the maintenance of human hair bulb either as an osmolyte [4,13,14,38] or a regulator of cysteine and/or calcium metabolism [35].

Alternatively, it is now well established that during the development of androgenetic alopecia and the progressive transformation of terminal into vellus-like follicles, inflammatory events occur that precede perifollicular fibrosis and ultimately sclerosis of collagenous streamers [15–17]. In this respect, taurine was described as a protective agent against age-related progressive renal fibrosis in rats [39]. As one of the master switches of the fibrotic program is TGF-β1, a growth factor known to inhibit hair growth *in vitro* [19], and since taurine can inhibit collagen synthesis [9], a downstream response to TGF-β1 [40], we further investigated a possible protective role of taurine against TGF-β1-induced alterations. Interestingly, the most conspicuous effect of TGF-β1 treatment was the curvature of hair follicle bulb, accompanied by a thickening of the dermal sheath as demonstrated by collagen staining. In fact, this phenomenon likely resulted from combined dermal sheath myofibroblast activation [41] and inhibition of matrix keratinocyte proliferation [19,42,43]. Taurine treatment dramatically counteracted this effect through a biological mechanism which remains to be explained.

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