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Effetti delle variazioni di gravità e delle radiazioni sulla ghiandola tiroidea

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ABSTRACT

Lo spazio è attualmente considerato la "nuova frontiera" per l'umanità. Oltre alla spinta naturale per l'ignoto, una caratteristica primordiale della natura umana, è stato previsto che la colonizzazione di altri pianeti potrebbe essere l'unica possibilità per l'umanità per sfuggire all'estinzione, il destino biologico altrimenti inevitabile per tutte le specie viventi.

Gli esseri umani, come altri organismi viventi sul pianeta Terra, si sono evoluti adattando le loro strutture e le funzioni biologiche del corpo per il campo gravitazionale della Terra, l'ambiente spaziale colpisce ogni funzione del corpo umano, questi cambiamenti possono causare gravi problemi di salute, sia nello spazio che al ritorno sulla Terra.

L'obbiettivo generale del lavoro è stato quello di valutare gli effetti della gravità e delle radiazioni sulla struttura/funzione della ghiandola tiroidea.

Lo scopo è stato quello di fornire indicazioni sulle possibili variazioni a livello funzionale e anatomico della tiroide nel corso delle missioni spaziali per il miglioramento delle condizioni di salute degli astronauti, essendo noto che tale ghiandola controlla il metabolismo di tutto l'organismo, influenzando così il corretto funzionamento del sistema cardiovascolare, osteotendineo, nervoso e immunitario.

Abbiamo pertanto valutato le modificazioni strutturali e funzionali della ghiandola in topi mantenuti in MDS, struttura sviluppata da Thales Alenia Space, per 91 giorni nella Stazione Spaziale Internazionale (ISS).

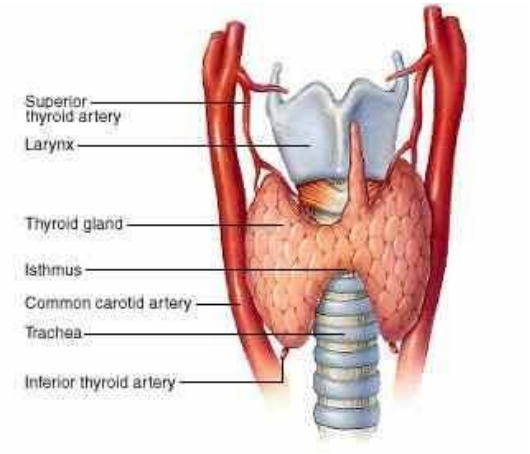
Dallo studio è emerso che nell'ambiente spaziale la ghiandola tiroidea subisce delle modificazioni sia nella sua componente follicolare e sia in quella parafollicolare partecipando in modo significativo ai danni riscontrati dagli astronauti al rientro dalle missioni spaziali.

I. INTRODUZIONE

1- LA TIROIDE

La **tiroide** è una ghiandola endocrina in grado di secernere e sintetizzare ormoni, che controllano numerose funzioni metaboliche, agiscono sullo sviluppo del sistema nervoso centrale e consentono l'accrescimento dell'organismo.

1.1 Anatomia e istologia



(Fig. 1)

La tiroide è costituita da due *lobi* connessi da un *istmo* ed è situata nel collo tra il secondo e il terzo anello tracheale, posteriormente ai muscoli sternoioideo e sternotiroideo, e inferiormente alla cartilagine tiroidea.

La grandezza dei due lobi è uguale nell'uomo e nella donna;(*Yildirim et al.,* 2006) ma in entrambi i

sessi il lobo destro è più grande rispetto al lobo sinistro (*Ying et al.,* 2009).

L'asse maggiore e quello minore dei due lobi sono differenti e le superfici delle aree sono rispettivamente 15.69 ± 0.9 and 11.52 ± 2.6 mm^2 (*Albi et al. 2012*).

La vascolarizzazione è sostenuta dalle arterie tiroidee superiori ed inferiori originate dalla carotide interna e dalla succlavia, mentre il drenaggio venoso confluisce nelle vene giugulari interne e succlavie (Fig.1). Entrambe le branche del sistema nervoso autonomo, ortosimpatica e parasimpatica, innervano la tiroide mediante terminazioni nervose distribuite sulle pareti vasali. Una sottile capsula fibrosa ricca di fibre elastiche riveste la tiroide e la suddivide in *lobuli* di dimensioni irregolari, a loro volta costituti da ***follicoli,*** strutture sferoidali di dimensioni variabili e da spazzi inter-follicolari.

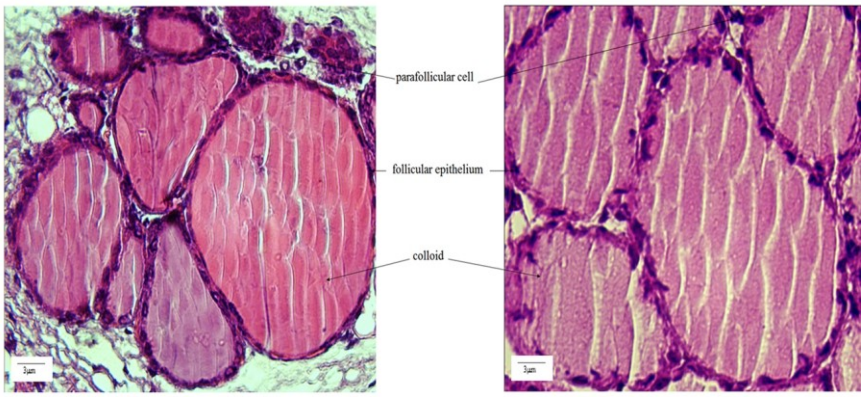
In condizioni fisiologiche i follicoli più grandi si trovano lungo il perimietro, mentre i più piccoli si trovano centralmente.

I follicoli rappresentano l'unità funzionale della tiroide e sono formati da un monostrato di tireociti (o cellule follicolari) che delimitano una cavità centrale. Il lume follicolare contiene una sostanza gelatinosa, la colloide, che è costituita principalmente da tireoglobulina (Tg) secreta dalle cellule tiroidee. (Fig. 2)

I tireociti sono cellule con netta polarità: il polo basale è adagiato sulla membrana basale, mentre il polo apicale delimita il lume follicolare in maniera irregolare per la presenza di numerosi microvilli. La stimolazione alla secrezione di ormoni tiroidei induce variazioni nella forma e nell'attività dei tireociti che dalla forma appiattita tipica della cellula quiescente, assumono un aspetto colonnare e dalla membrana apicale si dipartono estroflessioni pseudopodiche che inglobano la colloide permettendone il riassorbimento tramite pinocitosi.

Tra i follicoli, vi sono gli spazi interfollicolari, che comprendono *cellule parafollicolari* o *cellule C.*

Fig. 2



(Fig.2)

1.2 Fisiologia

1.2.1 Asse ipotalamo-ipofisi-tiroide

L'ipotalamo è la più importante regione del cervello coinvolta nella regolazione dell'asse tiroideo. Questo controlla il ritmo di produzione diurna dell'ormone di stimolazione della tiroide (TRH) e gioca un ruolo fondamentale nell'adattamento dell'asse ipotalamo-ipofisi-tiroide a fattori ambientali (Filers *et al*., 2014).

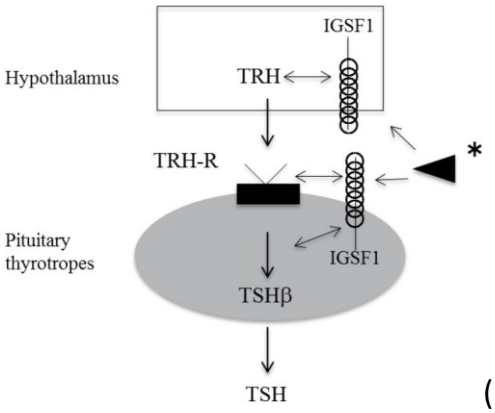
Infatti, l'adattamento a fattori ambientali è essenziale per la sopravvivenza di tutte le specie animali alle variazioni delle stagioni in termini di temperatura e disponibilità dei cibi.

1.2.2 TRH

Evidenze sperimentali hanno dimostrato che un cambiamento stagionale della disponibilità dell'ormone di regolazione della liberazione del TSH (TRH) nel cervello è un elemento cruciale per

l'adattamento. La liberazione del TRH è mediata dal controllo locale degli enzimi che metabolizzano gli ormoni tiroidei all'interno di cellule ependimali specializzate del terzo ventricolo dell'ipotalamo. Dentro queste cellule l'enzima degliodinasi Tipo2 è attivata in risposta alla lunghezza del giorno in estate convertendo il tetraiodio T4 in triiodiotironina (T3).

La disponibilità dell'ormone tiroideo nell'ipotalamo sembra essere un fattore importante nel guidare i cambiamenti fisiologici stagionali (*Wood et Loudon* 2014). (Fig.3)



(Fig.3)

1.2.3 Il TSH

La storia del TSH inizia quando nel 1926 Eduard Uhlenhuth dimostrò che la parte anteriore della ghiandola pituitaria era in grado di secernere una molecola in grado di stimolare l'attività tiroidea (*Uhlenhuth, 1927*).

Nei primi anni 70 la determinazione della struttura primaria delle subunità dell'ormone glicoproteico (*Persani et al., 1991*) e il clonaggio dei geni codificanti rispettivamente per la subunità α e β hanno

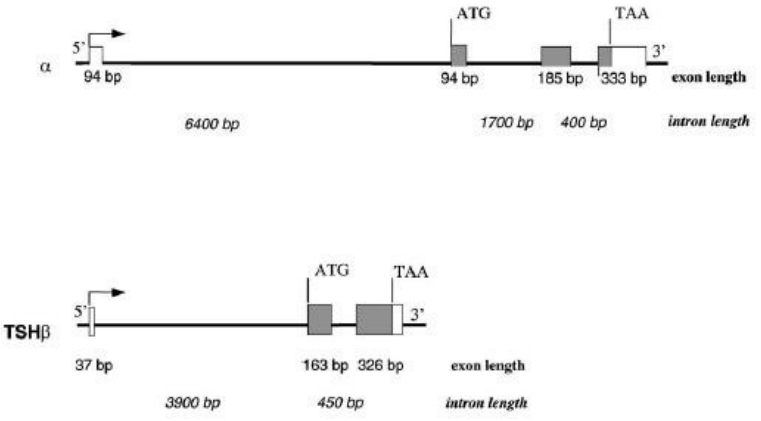
permesso di studiare l'espressione, la regolazione e l'attività della tireotropina (*Wondisford et al.. 1988*). Dopo aver identificato tramite cristallografia un'omologia con l'ormone hCG (*Wu et al., 1994*), si è constatato che il TSH espleta il suo effetto biologico legandosi ad una proteina posta sulla membrana plasmatica dei tireociti: il TSH recettore (*Pastan et al., 1966*).

1.2.3.1 TSH: chimica e biologia molecolare

TSH è una glicoproteina di 28 aminoacidi (30-kDa) sintetizzata e secreta dalle cellule tirotrope dell'ipofisi anteriore.

Appartiene alla superfamiglia degli ormoni glicoproteici (GpH) eterodimerici costituiti da una comune α-subunità e una β-subunità che conferisce specificità all'ormone stesso. Fanno parte di questa famiglia anche l'ormone follicolo-stimolante (FSH), l'LH e l'hCG (*Pierce and Parsons, 1981*).

L'α-subunità e la β-subunità sono codificate da geni localizzati rispettivamente sul cromosoma 6 e 1 (*Dracopoli et al., 1986*).



(Fig.4)

Il gene codificante per l'α-subunità è costituto da 4 esoni e 3 introni ed è lungo 9.4 Kb mentre il gene

codificante la β-subunità è costituito da 3 esoni e 2 introni e risulta essere circa la metà di quello codificante la subunità comune (4.9 Kb). Il primo esone è breve in entrambi i casi, non viene tradotto ed è separato dalla regione codificante da introne molto grande (Fig 4).

A differenza dell'α-subunità che presenta un'espressione più generale essendo comune a diversi ormoni glicoproteici, la β-subunità presenta un'espressione specifica solo al livello dell'ipofisi anteriore .

1.2.4 IL TSH RECETTORE

1.2.4.1 Il gene TSHr

Sfruttando l'elevata omologia tra i domini trans membrana dei GpHR, è stato possibile clonare il gene codificante per il TSHr nel 1989 (*Nagayama and Rapoport, 1991*). Il gene del TSHr è localizzato sul braccio lungo del cromosoma 14 (14q31) (*Libert et al., 1990*), si estende per oltre 60 Kb ed è costituito da 10 esoni (*Gross et al., 1991*).

Il gene codifica per una proteina costituita da 764 aminoacidi, costituita da un peptide segnale di 21 residui aminoacidici, un dominio extracellulare glicosilato formato da 394 residui e un dominio composto da 349 residui che costituiscono la porzione a sette domini trans membrana (7TMD), tipica dei GPCR (G protein coupled receptor), ed una corta coda citoplasmatica.

La struttura del gene è in stretta correlazione con quella della proteina: il dominio extracellulare, che costituisce la metà N-terminale del recettore, è codificato dai primi nove esoni, ciascuno dei quali codifica per uno o per un numero intero di motivi. La metà C-terminale del recettore, invece, contenente l'intero dominio trans membrana e la coda citoplasmatica, è codificata interamente dal

decimo esone.

Quest'ultimo sembra derivare da una vasta porzione priva di introni del gene codificante per gli altri GPCR. Da questa osservazione nasce l'ipotesi secondo la quale un gene codificante per l'antenato comune dei recettori per gli ormoni glicoproteici ipofisari sia stato originato dalla fusione di un gene, privo di introni, fortemente correlato agli altri GPCr, con un gene appartenente alla famiglia dei geni codificanti per proteine ricche di motivi a leucina. In seguito i geni di TSHr, LH/hCGr ed FSHr sarebbero risultati dalla triplicazione dell'antenato comune, seguita dalla dispersione su cromosomi differenti e dalla ulteriore evoluzione (*Gross et al., 1991*).

L'espressione del gene del TSHr non è limitata esclusivamente alla tiroide: l'mRNA e/o la proteina stessa sono stati individuati in numerosi tessuti e cellule quali linfociti, adipociti, fibroblasti retrooculari, cellule neuronali ed astrociti (*Crisanti et al., 2001; Mengistu et al., 1994 ; Davies et al., 2005*), tuttavia il significato fisiologico o patofisiologico di tale espressione extratiroidea non è ancora chiaro, benchè alcuni studi suggeriscano la possibilità di un ruolo di regolazione autocrina o paracrina del TSH sui linfociti (*Davies et al., 1994*) e un'azione di controllo negativo sulla formazione di osteoblasti ed osteoclasti.

1.2.4.2 Struttura primaria del TSHr

La struttura primaria del recettore, dedotta dal cDNA, conferma la sua appartenenza alla superfamiglia dei GPCr. Esso contiene sette segmenti con idrofobicità e lunghezza compatibili con quelle di segmenti trans membrana, inoltre alcuni di essi, in particolare il II ed il VII, hanno una forte

omologia di sequenza con i corrispondenti segmenti di altri GPCR. Il TSHr mostra alcune importanti differenze funzionali che lo contraddistinguono dagli altri recettori per gli ormoni glicoproteici (GpHR):

- sono presenti due segmenti (residui 38 – 45 e 316 – 366), che non hanno una controparte nei recettori per FSH o LH/hCG, localizzati nel dominio extracellulare (*Davies et al., 2005*). Il primo è fondamentale per il legame con il TSH e per l'interazione con anticorpi stimolanti il recettore, mentre il secondo è compreso tra due siti di taglio che danno origine a recettori dimerici. Alcuni recettori maturi, espressi sulla membrana cellulare, subiscono un taglio proteolitico, dando origine a due subunità, A e B, tenute insieme da ponti disolfuro, a livello del segmento 316 – 366 (*Rapoport et al., 1998*);

- analogamente al recettore per l'LH, la parziale degradazione proteolitica o piccole delezioni a carico del dominio extracellulare del TSHr ne determinano l'attivazione (*Van Sande et al., 1996; Zhang et al., 1995*). Il TSHr è, tuttavia, maggiormente soggetto a questo tipo di attivazione;

- l'affinità di legame per il TSH non viene diminuita dalla presenza di analoghi non idrolizzabili del GTP (*Akamizuet et al., 1994*);

Dal confronto della struttura primaria dei diversi GpHr, emerge che il dominio extracellulare mostra un'omologia di sequenza che raggiunge circa il 40%, mentre a livello del dominio trans membrana l'omologia è del 70% circa (*Vassartet al., 2004*). Questo pattern di omologia suggerisce che il legame specifico con l'ormone avviene a livello del segmento extracellulare, mentre il dominio contenente i sette segmenti trans membrana è responsabile della trasduzione del segnale tramite l'attivazione di proteine G_S o G_q.

Molti GPCr contengono residui di Serina o Treonina quali siti putativi di fosforilazione a livello delle anse citoplasmatiche (in particolare la III) o della corta coda citoplasmatica C-terminale (*Collins et al., 1991*). Tali siti sono implicati nella desensitizzazione del recettore in seguito all'esposizione al ligando. Il TSHr, al contrario, presenta pochi residui di Serina potenzialmente fosforilabili, spiegando la limitata desensitizzazione del recettore osservata in seguito alla stimolazione dei tireociti con TSH.

Studi recenti suggeriscono ulteriori meccanismi di trasduzione del segnale basati sulla β- arrestina, sulla GRK (G-protein-coupled receptor kinase) e sulle RGS (Regulators of G- protein Signaling) che, benché coinvolti primariamente nei meccanismi di desensitizzazione ed internalizzazione del recettore, sembrano responsabili della trasmissione del segnale dal recettore alla cascata delle MAPK (Mitogen-Activating Protein Kinase) (*Kursawe and Paschke, 2007*).

1.2.4.3 Struttura secondaria del TSHR

Il dominio extracellulare

Il dominio extracellulare del TSHr, responsabile del legame ad alta affinità del TSH, si compone di diverse strutture, tra le quali la più voluminosa è un cluster formato da 9 motivi ricchi di Leucina, le *Leucine-Rich Repeats* (LRRs) caratteristico dei GpHr (Fig. 5) Le LRRs sono comprese tra due domini ricchi in Cisteina denominati *Cysteine-rich Flanking Region* (CFRs**)** , importanti non solo per il legame con l'ormone ma anche per le successive modificazioni conformazionali che determinano l'attivazione del recettore (*Farid and Szkudlinski, 2004*).

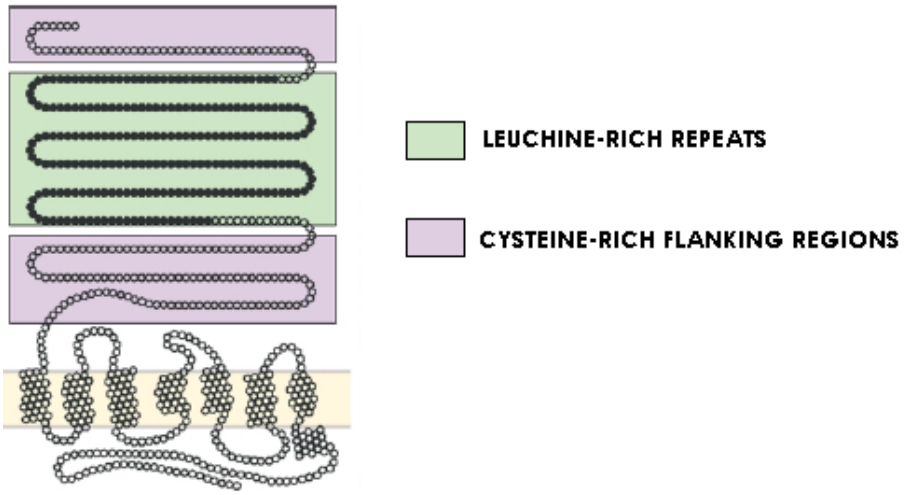


Figura 5 Rappresentazione bidimesionale della distribuzione di LLR e CFR all'interno del TSHR

L'ECD è, inoltre, composto anche da un linker strutturale (circa di circa 130 residui) tra le LRRs e il TMD, la cosiddetta "regione cerniera". Recenti studi hanno dimostrato che tale regione presenta non solo un ruolo strutturale, ma anche un ruolo funzionale nell'attivazione dei GpHr. (*Costagliola et al, 2002; Bonomi et al, 2006; Nurwakagari et al, 2007; Mueller et al, 2008; Kleinau and Krause, 2009*).

LEUCINE-RICH REPEATS (LRR): i dati resi disponibili per l'elaborazione di un modello tridimensionale sono stati ottenuti dallo studio dell'inibitore della ribonucleasi, la prima proteina contenente LRRs ad essere stata cristallizzata (*Kobe et al*., 1993). I LRRs sono motivi proteici costituiti da 20 – 25 aminoacidi, organizzati a formare un foglietto β e un'α-elica connessi da un'ansa. I nove LRRs presenti nel dominio extracellulare del TSHr sono disposti in modo sequenziale e formano una struttura a forma di ferro di cavallo, in cui i foglietti β delimitano la superficie interna concava responsabile del legame con il ligando (Fig. 6).

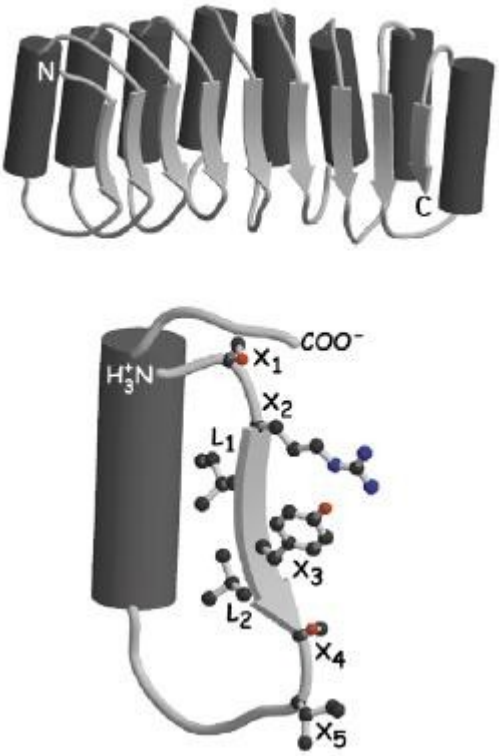


Figura 6: Le LRR sono costituite da 20-24 amminoacidi che formano un foglietto β seguito da un'α-elica. Il dominio ha la forma di un ferro di cavallo con i foglietti B che compongono una superficie concava. Ciascun foglietto β è costituito da residui amminoacidici i cui gruppi laterali sporgono all'interno della cavità mentre i residui di Leucina (L1 ed L2), idrofobici, sono importanti per la corretta conformazione proteica. [da Vassart, 2004]

Alcuni residui non leucinici, proiettati verso la cavità, sono responsabili della specificità di legame per l'ormone. Essi determinano una superficie che, mediante forze elettrostatiche attrattive e repulsive, è in grado di riconoscere specificatamente uno solo degli ormoni glicoproteici (TSH, LH/hCG o FSH) con alta affinità, impedendo in tal modo la stimolazione inappropriata del recettore.

A sostegno di questo modello sono stati condotti studi in cui, tramite mutagenesi sito-diretta, i residui non leucinici delle LRRs appartenenti al TSHr o al FSHr venivano sostituiti. I recettori mutanti mostravano la capacità di legare la hGC con un'affinità paragonabile a quella del recettore per l'LH/hCG, pur continuando a mantenere la capacità di riconoscere il proprio ligando naturale. Infatti, la sostituzione delle prime cinque LRRs del TSHr (aa 9- 165) con la regione corrispondente del LHr non ha mostrato alcun effetto sulla capacità del recettore di legare il TSH, indicando che le prime cinque

LRRs non determinano la specificità di legame (*Wonerow et al., 2001*). Nel caso del TSHr è risultato necessario effettuare dodici ulteriori sostituzioni amminoacidiche per ottenere un recettore in grado di riconoscere e legare con alta specificità esclusivamente l'LH/hCG (*Smits et al., 2003*). È inoltre nota una mutazione spontanea a carico della Lisina 183 (K183R), appartenente alla quinta LRR del TSHr, che determina un aumento della sensibilità del TSHR alla hCG. Questa causa una condizione di severo ipertiroidismo durante la gravidanza, quando, soprattutto durante il primo trimestre, i livelli di hCG sierici sono estremamente elevati (*Rodien, 1998*).

In aggiunta alle interazioni ormono-specifiche geneticamente codificate nella struttura primaria dei ligandi e delle LRRs dei recettori, è stata messa in evidenza anche l'importanza delle interazioni ioniche non ormono-specifiche che coinvolgono i residui tirosinici presenti nel dominio extracellulare del TSHr: la solfatazione del recettore a livello di alcune tirosine, e, probabilmente, i gruppi solfatati delle catene di carboidrati e di acido sialico, contribuiscono all'affinità di legame per l'ormone senza interferire con la specificità, tramite un' interazione diretta con il ligando o il mantenimento della forma funzionale del dominio extracellulare (*Bhowmick, 1996*).

CYSTEINE-RICH FLANKING REGIONS (CFR): Nonostante la struttura tridimensionale dei due domini non sia stata completamente determinata, è noto che entrambi sono coinvolti nel legame del TSH al dominio extracellulare del recettore. Si ipotizza che le CFR comprendano quattro α-eliche che formano una struttura compatta stabilizzata da interazioni non covalenti, con diversi segmenti che sporgono tra la seconda e la terza α-elica.

Una volta ancorato alla CFR, il TSH induce una serie di cambiamenti conformazionali che portano all'attivazione del recettore e delle vie di signaling o che aumentano l'accessibilità delle proteasi ai siti

di taglio o delle cellule immunocompetenti alle regioni riconosciute come epitopi (*Kackzor and Matosiuk, 2002*).

In aggiunta, i residui di cisteina distribuiti sulle estremità N- e C-terminali del dominio extracellulare e a livello delle anse extracellulari sono molto importanti sia per l'assunzione ed il mantenimento del corretto folding proteico sia per l'attivazione del recettore. Ci sono ben undici cisteine nel dominio extracellulare, che occupano le posizioni 24, 29, 31, 41, 176, 283, 284, 301, 390, 398 e 408, che potrebbero formare legami disolfuro necessari per la struttura terziaria del TSHr (*Farid and Szkudlinski 2004*). Ad esempio, la Cys41, appartenente al segmento 38 – 45 esclusivo del TSHr e rilevante nel legame con il TSH e con autoanticorpi stimolatori, è un residuo chiave per l'assunzione, da parte del TSHr, del corretto folding proteico in fase di sintesi e, quindi, dell'espressione in membrana del recettore, tramite la formazione di un legame disolfuro con Cys29 o Cys31 (*Farid and Szkudlinski, 2004*). Inoltre la formazione di tale legame comporta la formazione di un epitopo riconosciuto dagli autoanticorpi (*Song, 2001*).

1.2.4.4 Il dominio transmembrana e le anse intra ed extra cellulari

Il dominio transmembrana e le anse intra ed extracellulari, sono responsabili della conversione dello stimolo in un segnale intracellulare, tramite il reclutamento e l'attivazione di proteine G.

Benchè il TSHr umano possa associarsi con tutte le tipologie di proteina G (Gs, Gi, Gq/11, G12/13), l'attivazione della sola proteina Gs e, in minor grado, della proteina Gq/11, sembra necessaria per la regolazione della crescita e del differenziamento della tiroide e per l'induzione della sintesi ormonale

(*Vassart and Dumont, 1992*). Questo dato è basato sull'osservazione del rapido accumulo di cAMP intracellulare in seguito allo stimolo con una quantità di TSH da cinque a dieci volte minore di quella necessaria per attivare la via dell' inositolo trifosfato (*Van Sande et al., 1999; Van Sande et al., 1995*).

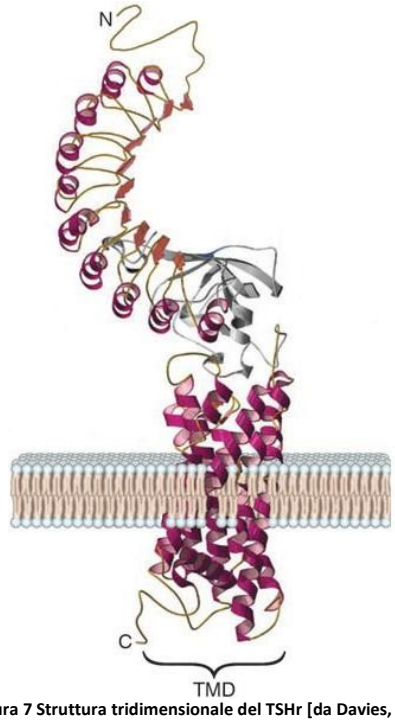


Figura 7 Struttura tridimensionale del TSHr [da Davies, 2005]

ANSE EXTRACELLULARI sono tre e sono implicate sia nel legame con l'ormone sia nella trasduzione del segnale all'interno dei tireociti. (Fig 7) Sono stati individuati alcuni aminoacidi chiave per entrambe queste funzioni. A livello della seconda e della terza ansa extracellulare sono presenti, rispettivamente, due residui di cisteina nelle posizioni 494 e 569, conservati in diversi GPCr: la formazione di un ponte disolfuro che mantenga le due anse posizionate correttamente è necessario perché il recettore sia in grado di trasdurre il segnale (*Gustavsson et al., 1994*). L'Asp 474 nella prima ansa extracellulare è necessario per la normale capacità di trasduzione del segnale da parte del recettore, mentre la sostituzione della Ile 486 con un residuo di Phe o Met (mutazioni attivanti) genera un recettore costituitivamente attivo, in grado di stimolare sia la via del cAMP, sia quella dell'IP3.

Altre tre mutazioni attivanti sono state descritte a livello della terza ansa extracellulare (Asn650Tyr, Val656Ala e una delezione di tre aminoaciacidi nel tratto di connessione tra la terza ansa ed il settimo segmento transmembrana –TM7): i loro effetti pro-attivanti sulla funzione recettoriale sono dovuti proprio alla stretta vicinanza e all'influenza sulla conformazione del sesto e del settimo segmento transmembrana, responsabili del mantenimento della forma inattiva del recettore.

SEGMENTI TRANSMEMBRANA (TMD). La porzione transmembrana del TSHr è importante per la stabilizzazione della conformazione inattiva del recettore, attraverso le interazioni che coinvolgono i residui amminoacidici dei vari TMD. Una sequenza consenso, altamente conservata fra i GPCR, è localizzata nel sesto segmento transmembrana (TM6) e contiene i residui amminoacidici che interagiscono con la terza ansa citoplasmatica del recettore.

Altre importanti interazioni coinvolgono il TM6 e il TM5 ed anche il TM6 e il TM7. La sostituzione dell'Asp 633, appartenente al TM6, rappresenta, infatti, la mutazione attivante del TSHR più frequente. Questo aminoacido forma un legame a idrogeno con l'Asn 674, appartenente al TM7, stabilizzando il recettore allo stato inattivo (*Wonerow et al., 2001*).

La natura debole di queste interazioni spiega la maggiore attività basale del TSHr rispetto a quella degli altri GPCr: l'isomerizzazione del recettore in una conformazione attiva necessita, probabilmente, di variazioni conformazionali minime (*Wonerow et al., 2001*).

ANSE CITOPLASMATICHE. Le tre anse citoplasmatiche sono coinvolte nell'associazione del recettore con le proteine G e nell'attivazione delle vie di trasduzione del segnale (*Chazenbalk et al., 1991*), in particolare la seconda ansa citoplasmatica sembra coinvolta nel reclutamento delle proteine G, mentre la terza è responsabile dell'attivazione ottimale delle proteine G (*Wess, 1998*).

Benchè siano note mutazioni puntiformi, che determinano l'attivazione costitutiva delle vie di trasduzione del segnale, a carico di alcuni aminoacidi compresi nella prima o nella seconda ansa citoplasmatica, è stato osservato che, per quanto riguarda la terza ansa, è la lunghezza di questa a determinare l'attivazione o meno delle proteine G. Infatti, le mutazioni attivanti che coinvolgono la terza ansa citoplasmatica sono delezioni a carico del segmento C-terminale di quest'ultima: la lunghezza dell'ansa influisce sulla conformazione del TM6 che, a sua volta, rende conto dell'attivazione delle proteine G associate (*Wonerow et al., 1998*).

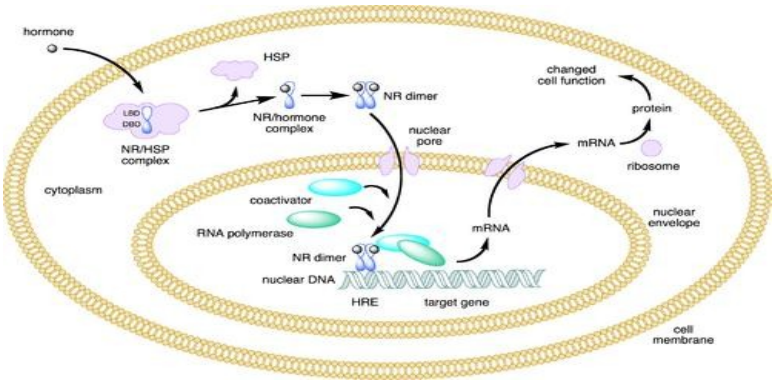
Sono stati condotti studi, tuttavia, che hanno messo in luce l'importanza, anche nella terza ansa citoplasmatica, di alcuni residui amminoacidici chiave non solo nella stabilizzazione della forma inattiva del recettore, ma anche nell'espressione in membrana e nell'efficienza di accoppiamento alle varie tipologie di proteine G.

1.2.5 Azione degli ormoni tiroidei a livello periferico

Gli ormoni tiroidei a livello cellulare interagiscono con i loro recettori per esplicare la loro funzione.
I recettori nucleari (NRs) appartengono ad una superfamiglia di fattori di trascrizione legati al DNA che possono essere attivati sia da steroidi sia da ormoni tiroidei o da altri metaboliti lipidici; (NRs) attivati dai ligandi possono regolare l'espressione genica legandosi ad elementi di risposta al DNA presenti nel promotore del gene target (*Mo and He*, 2014).
I meccanismi attraverso i quali il complesso T3–recettore nucleare modula la trascrizione genica sono solo in parte sconosciuti.
La sequenza centrale, che è altamente conservata in tutti i recettori nucleari, è deputata al legame con il DNA (DNA binding domain). Questa regione è ricca di cisteine che costituiscono due formazioni

digitiformi ciascuna stabilizzata da un atomo zinco, gli zinc fingers, necessari a riconoscere le sequenze di DNA specifiche per l'attivazione dei geni regolati (hormone response elements, HRE) e a stabilizzarne il legame.

La regione C-terminale che è responsabile del legame con l'ormone (hormone binding domain) contiene molti aminoacidi idrofobici che nella conformazione terziaria formano una tasca idrofobica in cui si impegna l'ormone. La regione N-terminale, che dimostra un basso grado di omologia viene quindi definita come ipervariabile e sembra essere principalmente coinvolta nell'attivazione dei meccanismi di trascrizione. In seguito a presentazione dell'ormone glicoproteico, i recettori nucleari tendono a dimerizzare legandosi ad un singolo *HRE*.



Le sequenze specifiche del DNA che costituiscono le HRE sono generalmente localizzate su segmenti che precedono dal lato 5' il promotore della RNA polimerasi del gene inducibile dagli ormoni. Queste sequenze vengono considerate enhancers in quanto in grado di aumentare l'attività trascrizionale del gene in questione.

Gli ormoni tiroidei intervengono quindi generando:

- *effetti sullo sviluppo neuronale e scheletrico fetale*: sebbene gli ormoni tiroidei siano in grado di attraversare la barriera placentare mediante i trasportatori MCT8 e MCT10 (*Loubière*, 2010), la tiroide fetale deve provvedere alla sintesi degli ormoni stessi per un corretto bilancio ormonale. Questo processo inizia sin dalla 11^{a} settimana di gestazione. Il difetto di T_3 nella vita fetale porta al quadro di cretinismo;

- *effetti sul consumo di ossigeno*: il livello di ossigeno a riposo è aumentato in presenza di elevati livelli di ormoni tiroidei e viceversa;

- *effetti sul sistema cardiovascolare*: gli ormoni tiroidei hanno un effetto cronotropo e inotropo sul cuore. Questo fenomeno può in parte essere attribuito al fatto che alti livelli di ormoni provocano un aumento dei recettori β-adrenergici nel muscolo cardiaco;

- *effetti emopoietici*: gli ormoni tiroidei causano un aumento dell'eritropoiesi probabilmente collegato all'aumento del consumo di ossigeno; gli ormoni tiroidei inducono, inoltre, la differenzazione delle cellule staminali pluripotenti verso cellule emopoietiche attraverso la produzione di fattori pluripoietin-like (*Sainteny et al*., 1990).

- *effetti sul sistema endocrino*: Gli ormoni tiroidei promuovono la differenziazione endocrina (*Virgine et al*., 2014)

 effetti sull'osso: gli ormoni tiroidei stimolano la differenzazione degli osteoclasti

con un meccanismo dipendente dall'interazione dell'attivatore recettoriale del ligando del fattore nucleare kappaB (RANKL) con RANK (*Kanatani et al,*2004)

1.2.6 Follicolo

Il follicolo tiroideo è adibito alla produzione degli ormoni tiroidei triiodiotironina (T3) e tetraiodiotironina (T4).

La sintesi degli ormoni avviene all'interno dei tireociti a partire dalla tireoglobulina, glicoproteina caratterizzata dalla presenza di residui tirosinici che possono venir iodati dopo ossidazione dello ioduro.

Da tale reazione si ha la formazione di monoiodiotirosina o di iodiotirosina; la combinazione di una monoiodiotirosina e una diiodiotirosina porta alla formazione di T3 mentre la combinazione di due diiodiotirosine porta alla formazione di T4.

La T_4 presente in circolo deriva esclusivamente dalla tiroide, mentre la maggior parte della T_3 circolante è di origine extratiroidea, derivando dalla desiodazione della T_4 nei tessuti periferici.

Nel plasma i due ormoni circolano in gran parte legate a proteine di trasporto degli ormoni tiroidei: la TBG, una globulina specifica (*Thyroid Hormone Binding globulin*), la TBPA (*thyroxin binding prealbumin*) e l'albumina. La TBG è una glicoproteina presente in quantità scarse nel plasma e ha un unico sito di legame per la T_4 o per la T_3 ma presenta un alto grado di affinità per entrambi gli ormoni. La TBPA è presente in concentrazioni medie, non lega in maniera apprezzabile la T_3, mentre lega circa il 15% della T_4 per la quale ha una bassa affinità.

L'albumina ha una scarsa affinità per gli ormoni tiroidei e nonostante ciò lega circa il 40% della concentrazione totale di T_3 e T_4. Il legame tra ormone e proteina di trasporto avviene secondo la legge di azione di massa e, in quanto tale, è reversibile. Ne deriva che un aumento delle proteine di trasporto determina all'inizio una diminuzione transitoria della quota libera, e in seguito, da una fase di ripristino dei suoi livelli originari. Solo la frazione libera degli ormoni tiroidei è biologicamente attiva in quanto può penetrare nella cellula bersaglio.

La T_4 libera, previa desiodazione a T_3, oppure direttamente la T_3 libera, interagendo con il recettore nucleare esplicano il loro effetto biologico e vengono quindi metabolizzate.

Benché gli ormoni tiroidei siano altamente liposolubili, il loro passaggio nel compartimento citoplasmatico della cellula bersaglio avviene con un meccanismo probabilmente più complesso della semplice diffusione. L'effetto biologico degli ormoni tiroidei è mediato dall'attivazione di un recettore nucleare con affinità per la T_3.

Un aumento della produzione di ormoni viene associato all'aumento di follicoli ma non all'aumento del loro volume (*M.L. Hartoft-Nielsen 2005).*

1.2.7 Spazio interfollicolare

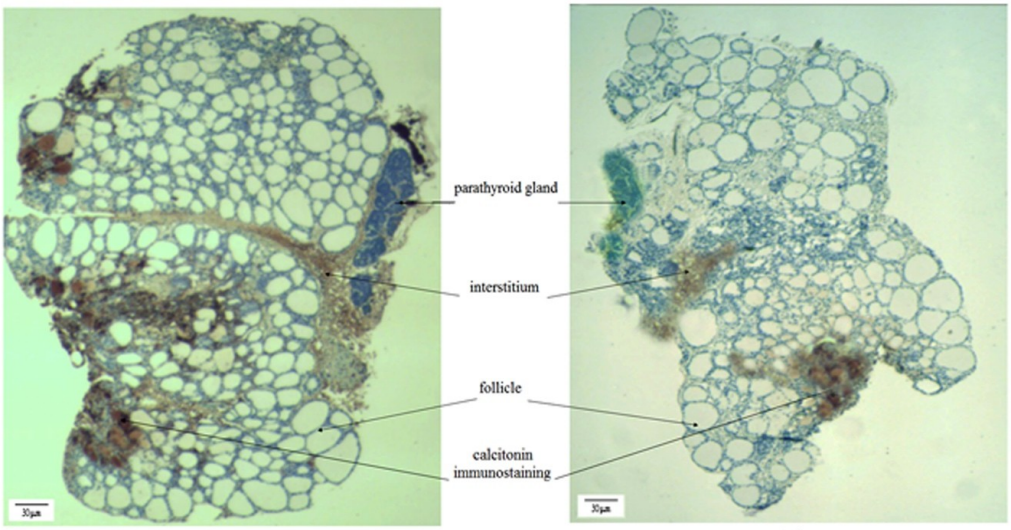
Le cellule parafollicolari si trovano disposte in piccoli cluster lungo la zona periferica dei follicoli, prevalentemente nella parte centrale dei lobi tiroidei. La loro funzione principale è la produzione di calcitonina, ormone coinvolto nella regolazione del bilanciamento calcio/fosforo (*Hoyes et al*,. 1985).

Nonostante le cellule parafollicolari o cellule della tiroide C sono generalmente note per la produzione di calcitonina, è stato messo in evidenza il loro ruolo nella produzione di numerosi peptidi di regolazione, come somatostatina e grelina (*Ahrén*, 1991), catacalcina I, catacalcina II, con rilascio di

gastrina, tiroliberina e (helodermin) (*Sawicki*, 1995). Inoltre le cellule C, regolate dal TSH a causa dell'espressione del TSHr, sono coinvolte nell'asse ipotalamo-ipofisi-tiroide (*Morillo-Bernal et al*., 2009). Ricerche effettuate hanno mostrato che le cellule C esprimono TRH, svolgendo attività paracrina sulle cellule follicolari e inducendo in loro l'espressione del TRHr (*De Miguel et al*.,2005). In questo modo le cellule C sono responsabili della regolazione intra-tiroidea delle cellule follicolari permettendo un'interrelazione tra le due popolazioni endocrine (*Martín-Lacave et al*.,2009).

È stato dimostrato che la produzione di calcitonina è differente nel lobo destro e nel lobo sinistro della tiroide. (*Albi et al*.,2012). (Fig 9)

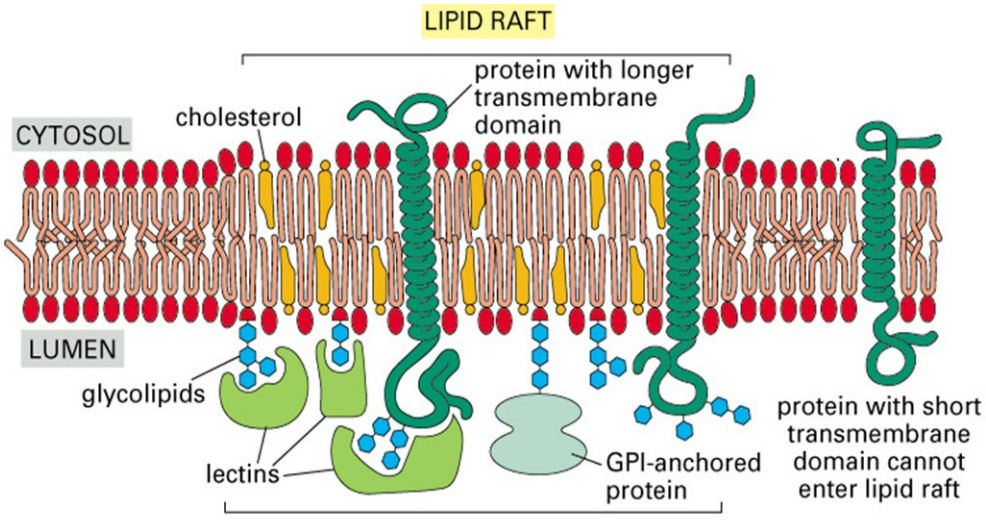
Fig.1



(Fig 9)

2- Rafts Lipidici

Dalla formulazione dell'ipotesi dei rafts la ricerca ha speso molte risorse nell'identificazione e descrizione di questi microdomini lipidici associati a tipi cellulari differenti (*Maggi et al*., 2002; *Nebl et al*., 2002; *Patel et al*., 2001; *Leshchyns'ka et al*., 2013).



(Fig 10)

Dopo anni di studio, è stato generalmente assunto che la membrana cellulare possa essere suddivisa in regioni organizzate, rafts lipidici e regioni non organizzate.

I rafts lipidici sono microdomini di membrana ad alto contenuto di colesterolo, glicosfingolipidi e fosfolipidi dalle catene di acidi grassi sature; sono caratterizzati da una bassa densità e sono resistenti ai detergenti (*Schuck et al*., 2003; *Slimane et al*., 2003).

Studi effettuati hanno portato a considerare che circa il 13% della membrana è costituita da microdomini lipidici (*Schutz et al*., 2000; *Hao et al*., 2001).

La composizione biochimica dei rafts lipidici è una delle proprietà caratterizzanti di questi microdomini; molte ricerche sono state portate avanti analizzando rafts lipidici estratti da diversi tipi cellulari con diverse tecniche per poter riuscire a descrivere una composizione chimica di queste strutture.

In generale questi studi hanno dimostrato come i rafts lipidici sono arricchiti in colesterolo e glicosfingolipidi ma relativamente poveri di glicerolfosfolipidi; in particolare ricerche fatte su cellule MDCK hanno mostrato come i rafts lipidici presentino un incremento notevole nel contenuto in colesterolo (CHO) e sfingomielina (SM) rispetto alla composizione della membrana (*Brown and Rose*, 1992).

In particolare il contenuto in CHO e SM è rispettivamente 32mol% e 14mol%, notevolmente più alto di quello riscontrato in membrane cellulari intere (*Prinetti et al*,. 2000).

È interessante osservare anche i dati relativi all'analisi delle catene di acidi grassi associate ai fosfolipidi contenuti in rafts. In diversi tipi cellulari è stato visto come il tasso di catene acide sature o monoinsature passa dal 45 %, riscontrato nella membrana plasmatica, al valore di 60% riscontrato a livello solo dei rafts (*Fridriksson et al*,. 1999; *Pike et al*., 2002).

Oltre alla conformazione caratteristica lipidica, i lipid rafts hanno anche delle proteine associate caratteristiche: Caveolina, proteine GPI-ancorate, proteine doppiamente elicate, G-proteins a basso peso molecolare ed eterotrimeriche, chinasi della famiglia SRC, recettori PGDF ed EGF, MAOP chinasi e protein chinasi C (*Smart et al*., 1999; *Song et al*., 1996; *Mineo et al*.,1996; *Nickel et al*., 1997; *Brown*

and Rose, 1992).

Le ricerche hanno associato a queste strutture diverse funzioni in importanti processi cellulari quali la trasduzione del segnale, endocitosi, traffico intracellulare di proteine e lipidi, e la trasmissione e lo sviluppo di diverse malattie (*Van der Goot and Harder*, 2001). Nei processi di trasduzione del segnale grande importanza viene rivestita dalle proteine della famiglia STAT (Signal Trasducer and Activators of Trascription), in particolare dalle forme STAT1 e STAT3. La famiglia è costituita da sei proteine citoplasmatiche coinvolte nella trasduzione del segnale e nell'espressione di diversi geni implicati nel differenziamento cellulare. In particolare le isoforme 1 e 3 sono proteine ubiquitarie, coinvolte nell'azione intracellulare dei recettori tirosinchinasici e di quelli delle citochine. In quest'ultimo caso la loro attivazione avviene mediante la fosforilazione ad opera di chinasi citoplasmatiche della famiglia JAK (*Clementi and Fumagalli*, 1996). Queste proteine, una volta attivate, traslocano nel nucleo stimolando la trascrizione genica. È stato visto che i rafts lipidici svolgono un ruolo essenziale nell'attivazione delle proteine STAT, tanto che è possibile parlare di una "raft-STAT signaling hypothesis". Esperimenti specifici hanno confermato l'ipotesi mostrando come il 10% delle molecole di STAT1 e STAT3 presenti nella cellula risieda appunto su microdomini lipidici (*Sehgal*, 2003).

I rafts lipidici sono stati visti anche come sede di interazione di un vasto numero di patogeni come ad esempio il virus dell HIV-1, infatti i glicosfingolipidi localizzati sulla superficie dei rafts facilitano sia la fusione del virus sulla membrana che la stabilizzazione del complesso CD4-gp120, favorendo inoltre il rilascio dei virioni neosintetizzati e la loro diffusione nell'organismo (*Djordjevic et al*., 2004).

Altri virus come l'ebola, Epatite C e Marburg sono in grado di infettare le cellule attraverso l'interazione con rafts lipidici (*Panchal et al*.,2003; *Aizaki et al*., 2004).

È interessante anche il coinvolgimento dei microdomini lipidici nella genesi di malattie degenerative quali il morbo di Alzheimer. Associate ai rafts sono presenti proteasi che svolgono un ruolo centrale nella produzione di fibrille amiloidee, studi molto recenti hanno inoltre evidenziato che tra i fattori di rischio nello sviluppo dell'Alzheimer vi sia la diffusione del virus dell' Herpes simplex 1nel SNC. Esso sarebbe in grado di penetrare a livello neuronale integrando con proteine espresse sulla superficie dei rafts lipidici.

L'utilizzo di statine in grado di abbassare i livelli di colesterolo sulle membrane e quindi di favorire una destabilizzazione dei rafts lipidici, sembrerebbe diminuire il rischio di Alzheimer (*Hill et al*.,2005).

2.1 Rafts Lipidici e Tiroide

In condizioni normali il TSHr si trova nei rafts lipidici e il complesso raft-TSHr è regolato dal TSH. Dopo l'esposizione al TSH con conseguente trasduzione del segnale e l'attivazione cellulare i complessi rafts-TSHr scompaiono dalla superficie delle cellule, probabilmente perché l'ormone stimola la formazione di monomeri, permettendo la loro rapida uscita *(Latif et al*.,2003).

Gli stessi autori nel 2007 dimostrano che la forma monomerica dei recettori esiste sia nei rafts e sia su frazioni non rafts della membrana cellulare mentre la forma multimerica è situata preferibilmente nei rafts (*Latif et al*.,2007).

La dimostrazione della localizzazione del TSHr nei rafts di membrana è stata dimostrata con l'uso dell' insetticida DDT; infatti tale sostanza è in grado di alterare la struttura dei rafts di membrana con conseguente perdita del TSHr (*De Gregorio et al*., 2011).

Inoltre Martin Belmonte et al., hanno dimostrato che anche la tireoglobulina è associata ai rafts di membrana poiché hanno trovato che tale proteina è localizzata nei rafts della membrana plasmatica apicale dei tireociti (*Martin Belmonte et al*., 2000).

3- Ipogravità ed Ipergravità

Lo spazio è attualmente considerato la "nuova frontiera" per l'umanità. Oltre alla spinta naturale per l'ignoto, una caratteristica primordiale della natura umana, è stato previsto che la colonizzazione di altri pianeti potrebbe essere l'unica possibilità per l'umanità per sfuggire all'estinzione, il destino biologico altrimenti inevitabile per tutte le specie viventi.

Gli esseri umani, come altri organismi viventi sul pianeta Terra, si sono evoluti adattando le loro strutture e le funzioni biologiche del corpo per il campo gravitazionale della Terra, l'ambiente spaziale colpisce ogni funzione del corpo umano; anche se questi cambiamenti possono causare gravi problemi di salute, sia nello spazio che al ritorno sulla Terra, nella maggior parte dei casi, i loro effetti sono solo temporaneamente invalidanti e, dopo il loro ritorno sulla Terra, gli astronauti di solito riescono a riadattarsi rapidamente all'ambiente e alla gravità Terrestre (*Mulavara et al*.,2010).

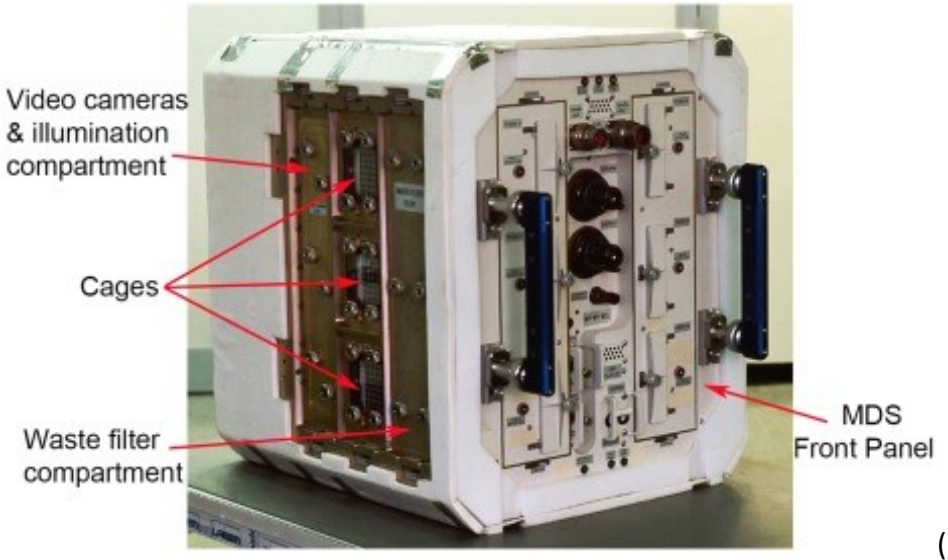
Durante le missioni spaziali, gli astronauti sono separati dalla loro vita quotidiana, e costretti a vivere in uno spazio limitato per lunghi periodi di tempo (*Pálinkás*, 2001); si ritiene generalmente che la partenza rappresenti una condizione di potenziale pericolo per lesioni fisiche acute e/o traumatiche (*Yue et al*., 2012), induce la riduzione dell'udito e dell'equilibrio (*Dechesne et al*., 1993), cambia l'umore e l'attività corticale del cervello (*Schneider et al*., 2010), le prestazioni mentali (*Manzey e Lorenz*, 1998), e l'attività elettrica cerebrale (*Lorenz et al*., 1996). Poco è noto circa gli effetti di

confinamento sull' equilibrio del corpo per lunghi periodi di tempo, che, in tali condizioni, è molto probabile che sia soggetto a perturbazioni significative nello spazio, come già riferito in relazione alle variazioni patologiche nelle funzioni corporee degli astronauti (*Rowe*, 2009; *Smith et al*, 2012;. *Mermel*, 2013).

E 'generalmente accettato che un' elevata forza di accelerazione gravitazionale che agisce lungo l'asse del corpo dalla testa ai piedi (+ Gz) provoca notevoli sollecitazioni sui vari organi, compreso il cervello, il cuore, reni e fegato. Gli astronauti durante le missioni spaziali sono sottoposti ad iper-gravità durante la fase di lancio, benchè questa sia una fase transitoria e non di lunga durata è stato visto che tale condizione può indure cambiamenti a livello fisiologico, infatti l'esposizione a iper-gravità ha dimostrato di ridurre fortemente il flusso di sangue agli organi viscerali, tra cui i reni, milza, pancreas e fegato. (*Kim et al*., 2014).

(Fig.11)

Nel 1998 il comitato scientifico dell'Agenzia Spaziale Italiana (ASI) seleziona il programma MDS (Fig.12), lo scopo originale del programma è stato quello di sviluppare una struttura per condurre ricerche in condizioni di microgravità sulla formazione ossea e di sviluppare contromisure specifiche per l'osteoporosi. Tuttavia, il programma MDS è stato sviluppato, da Thales Alenia Space - Italia (stabilimento di Milano, Italia), una società di impianti di sviluppo per il sostegno di sperimentazione scientifica nello spazio, come una struttura di uso generale che può essere adattato e utilizzato nella ISS da un grande numero di scienziati per esperimenti, con i topi, di lunga durata in diverse aree di ricerca. Infatti, nella configurazione standard, MDS può accogliere 6 topi fino a 100 giorni con possibile estensione a 180 giorni a bordo della ISS (*Ranieri Cancedda et al*.,2012).



(Fig. 12)

II. SCOPO DEL LAVORO

L'obbiettivo generale del lavoro è stato quello di valutare gli effetti della gravità e delle radiazioni sulla struttura/funzione della ghiandola tiroidea.

Lo scopo è stato quello di fornire indicazioni sulle sue possibili variazioni nel corso delle missioni spaziali per il miglioramento delle condizioni di salute degli astronauti. È infatti noto che la tiroide controlla il metabolismo di tutto l'organismo influenzando così il corretto funzionamento del sistema cardiovascolare, osteotendineo, nervoso e immunitario. Dunque, un'alterazione della ghiandola tiroidea potrebbe essere implicata nella maggior parte dei danni riportati dagli astronauti al rientro delle missioni spaziali. Gli obbiettivi specifici sono stati quelli di valutare in condizioni di ipogravità:

1- Le variazioni strutturali della ghiandola tiroidea;

2- Il comportamento della Galectina-3 come marker tumorale dei tireociti;

3- Le possibili alterazioni molecolari tiroidee.

4- Il comportamento della calcitonina;

5- Quanto il confinamento possa essere la concausa dei danni riportati al rientro delle missioni spaziali;

6- Confrontare danni da ipogravità con quelli da ipergravità;

7- Valutare l'effetto dei fasci di protoni accelerati sui tireociti

III. MATERIALI E METODI

1- Materiali

1.1 Standard e reagenti

PC, SM, non-idrossi acido grasso ceramide, phenylmethylsulfonylfluoride, acetonitrile, metanolo, 2-propanolo, metil-tert-butil etere (MTBE), acido formico, cloroformio, colesterolo (CHO), TSH sono stati ottenuti da Sigma Chemical Co. (St. Louis, MO, USA).; Piastre TLC (gel di silice G60) sono stati acquistati da Merck (Darmstadt, Germania); il radioattivo [Me-14C] SM (54,5 Ci / mol, 2.04 GBq / mmol), [Me-3H] (l-3-phosphatidyl- [N-Me-3H] -Colina-1,2-dipalmitoil, 81.0 Ci / mmol, 3,03 TBq / mmol), Kit di chemiluminescenza sono di Amersham Pharmacia Biotech (Rainham, Essex, Regno Unito); EcoscintA National Diagnostic (Atlanta, GA, USA.). Anticorpo Anti-Bax è stato ottenuto da Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). anti-STAT3, anti-SMasi e anti-SM-sintasi anti-TSHr, anti-Caveolina e anticorpi secondari per immunoblotting e anticorpi secondari per immunoflorescenza FITC, Tric. SDS-PAGE peso molecolare standard era di Bio-Rad Laboratories (Hercules, CA, USA). SM 18: 1 12: 0, SM 18: 1 16: 0, SM 18: 1 18: 1, SM 24: 0, ceramide 18: 1 16: 0, ceramide 18: 1 18: 0, ceramide 18: 1 20 : 0, ceramide 18: 1 24: 0 sono stati acquistati da Avanti (Avanti Polar, Alabaster, AL, USA). Kit cAMP Cabru sas (Milano, Italia.). Anticorpi anti-HBME-1 Ki-67 (clone MIB1) Dako (Milano, Italia). Anticorpi anti-CK19 e Anti-Galactina3, Anti-NCL-L-Calcitonina sono della Leica Biosystems (Newcastle, ltd, UK)

1.2 Animali

Sono stati utilizzati topi maschi C57BL/10J di 8 settimane di vita.

1.3 Cellule FRLT-5

Le cellule sono state preparate e caratterizzate nel laboratorio Ambesi-Impiombato *(Albi et al*., 2010).

Le cellule sono state coltivate in Ham F-12 modificato con siero di vitello al 5% e 6 ormoni: 10 ng/mL glycil-l-istidil-l-lisina acetato (Sigma), 10-8 M idrocortisone (Sigma), 10 mg/ml di insulina (Sigma), 10 mg / mL somatostatina (Sigma), 5 mg/ml transferrina (Sigma), 10 mU/mL TSH (Sigma). Le FRTL-5 sono stati mantenute a 37 °C al 5% di CO2 e 95% di umidità. Le cellule coltivate in presenza di TSH che stimola la proliferazione cellulare sono state chiamate cellule TSH+, mentre le cellule coltivate senza TSH sono state chiamate cellule TSH-. Le cellule sono state contate e seminate alla concentrazione 5×10^5/60 mm per piastra con di TSH+ per consentire l'adesione. Dopo 24h le cellule sono state lavate e le colture continuate per 7 giorni in un mezzo TSH+ oTSH-. Per l'esperimento le cellule FRTL-5 proliferanti e quiescenti sono state raccolte e poi seminate ad una densità di $1x10^6$/60 mm in piastre con 1 mL TSH + o TSH- media per 24 ore in incubatore per consentire l'adesione e quindi ciascuna piastra, è stata posta all'interno di scatole idonee per la coltivazione, dove la temperatura è stata fissata a 37 °C. Dopo la semina in piastra, le cellule sono state sottoposte a fasci di protoni prodotti e accelerati da acceleratori dal CERN (Ginevra, Svizzera). Sono stati usati Acceleratori con radiofrequenza lineare come iniettori per sincrotroni e come acceleratori autonomi per la produzione di intensi fasci di particelle, grazie alla loro capacità di accelerare correnti di fasci ad alte velocità di ripetizione. Le cellule sono state utilizzate in parte per la preparazione dei lisati liberi dai nuclei (NFL) e

nuclei purificati (N), e in parte per l'analisi della proteina apoptotica Bax. Le cellule sono state lavate due volte con PBS e centrifugate a 800 g per 10 min. Il pellet è stato sospeso in tampone ipotonico (1,5 M di saccarosio, 3 mM CaCl2, 2 mM Mg acetato, ditiotreitolo 0,5 mM, 1 mM PMSF, 3 mM Tris-HCl pH 8,0, 1 ml/106 cellule) e delicatamente omogeneizzate con omogeneizzatore teflon-vetro. Parte del omogenato è stato centrifugato a 500 g per 30 minuti a 4 ° C per NFL e parte è stato utilizzato per la preparazione e l'isolamento dei nuclei. A tal fine le cellule omogeneizzate sono state trattate con 1% Triton X-100 in tampone ipotonico (0,5: 1 v/v); la sospensione cellulare è stata agitata su vortex per 30s ed è stato aggiunto il buffer, contenente 1,5 M di saccarosio, (0,25: 1 v/v). Dopo centrifugazione a 2000g per 10 min il pellet contenente nuclei è stato lavato due volte con soluzione Barnes et al., 0.085 M KCl, 0,0085 M NaCl, 0,0025 M MgCl2, tricloroacetico-HCl 0,005 M, pH 7,2).

2- Metodi

2.1 Esperimenti in Ipogravità

Per l'esperimento spaziale sono stati utilizzati 3 topi WT e 3 Pleiotropina (PTN) TG, i quali sono stati singolarmente alloggiati nel MDS, di dimensioni 11,6 × 9,8 × 8,4 cm; cibo e acqua sono stati forniti ad libitum. L'MDS è stato lanciato nello Space Shuttle Discovery all'interno dello spazio Transport System (STS), il 28 agosto 2009.

Soltanto 1 topo WT e 2 topi TG sono ritornati vivi dopo il volo spaziale durato 91 giorni; dopo l'uccisione dei topi mediante anidride carbonica presso l'impianto di Life Sciences Supporto di Kennedy Space Center, sono stati prelevati gli organi entro 3-4 ore dopo l'atterraggio e congelati immediatamente secondo i vari protocolli sperimentali. La procedura è stata approvata dal protocollo n ° IACUC FLT-09-070 (KSC).

Contemporaneamente sono stati effettuati due esperimenti di controllo in animali della stessa specie, dello stesso sesso ed età: 1) 3 WT e 3 TG sono stati mantenuti presso il Vivarium del Centro di Biotecnologie Avanzate di Genova, Italia; 2) 3WT e 3 TG della sono stati mantenuti in MDS a terra per 3 mesi.

Quantità di cibo e di acqua e le condizioni ambientali sono state simulate come nel gruppo di volo. Dopo 3 mesi i vari organi sono stati campionati da entrambi i gruppi di topi controllo (Ranieri Cancedda et al.,2012).

In tutte le fasi dell'esperimento (pre-volo, durante il volo e post-volo) il trattamento degli animali era

in conformità con i principi espressi nella "Guida per la cura e l'uso di animali da laboratorio" (Ufficio di Scienza e Salute Reports del National Institute of Health USA, Bethesda, USA). L'approvazione di questo esperimento MDS è stato richiesto e ottenuto dal Comitato americano istituzionale (IACUC) con protocollo n ° FLT-09-070 (KSC), nonché dal Comitato Etico del Fondo Animal dell'Istituto Nazionale per la Cancer Research (Genova, Italia) e dal Dipartimento di Sanità Pubblica veterinaria del Ministero della Salute italiano (protocollo n ° 4347-09 / 03/2009-DGSA.P.).

2.2 Esperimenti di Ipergravità

Tre topi WT e tre TG della stessa razza, sesso ed età di quelli usati negli esperimenti di ipogravità, sono stati mantenuti in ipergravità con condizione simile all'esperimento MDS, in una centrifuga 2g nel laboratorio del DR. Yohira all' Osaka University, Osaka, Giappone.

I topi controllo sono stati preparati come quelli riportati negli esperimenti di ipogravità.

Gli animali sono stati trattati e le tiroidi sono state scisse e processate con le stesse procedure usate negli esperimenti di ipogravità.

2.3 Trattamento tessuto tiroideo

I lobi tiroidei sono stati fissati in una soluzione di Formaldeide 4% in tampone fosfato per 24h. Le tiroidi sono state incluse in paraffina e i blocchi di paraffina sono stai sezionati effettuando sezioni

dello spessore di 4-µm. Le sezioni sono state poste su vetrini di vetro specifici. Ciascun vetrino conteneva una coppia di sezioni ad un ugual distanza di 140 µm; sono state campionate tra 7 e 14 coppie di sezioni escludendo la prima e l'ultima e sono state poi utilizzate per l'analisi morfologica immunoistochimica e di immunoflorescenza.

Per l'analisi le sezioni sono state deparaffinizzate e reidratate con lavaggi di xilene ed etanolo a concentrazione progressivamente decrescente.

2.4 Analisi morfologica

Le sezioni sono state colorate con ematossilina-eosina (Chroma-Gesellschaft, Germania) e analizzate utilizzando il microscopio invertito Euromex FE 2935 (ED amhem, Olanda), fornito del sistema di telecamera CMEX 5000.

2.5 Analisi immunoistochimica

Per l'analisi immunoistochimica la soluzione Bond Dewax è stata utilizzata per rimuovere la paraffina dalle sezioni prima della reidratazione e immunocolorazione con il sistema automatizzato Bond (Leica biosystems newcastle Ltd, Inghilterra). L'immunocolorazione è stata effettuata in accordo con Bancroft and Stivens (1996) utilizzando specifici anticorpi e le osservazioni sono state effettuate utilizzando il microscopio soprariportato.

2.6 Analisi ad immunofluorescenza

Le sezioni di tessuto sono stati deparaffinate e reidratate attraverso una serie lavaggi con xilene ed etanolo. Dopo 3 lavaggi con tampone fosfato salino (PBS), le sezioni sono state incubate tutta la notte in PBS a 4 °C con 2ug/ml di anti-anticorpi primari TSHr diluiti in una soluzione allo 0,5% di albumina di siero bovino. I vetrini sono stati lavati 3 volte con PBS e incubati con anticorpi secondari coniugati a fluorocromi FITC per 1 ora a temperatura ambiente. Dopo 3 lavaggi con PBS, i vetrini sono stati montati con glicerolo e lamelle. I campioni sono stati esaminati con un microscopio a fluorescenza (Olympus IX 51) munito di sistema 50 Olympus DP e analizzati a 20x.

2.7 Determinazione delle proteine

La determinazione delle proteine è stata eseguita il metodo di *Lowry* (*1951*), modifica del metodo colorimetrico di Folin-Ciocalteau (*Folin, 1927*).

I valori proteici dei campioni in esame sono stati calcolati sulla base di una curva di standard di riferimento costruita utilizzando concentrazioni scalari di albumina serica bovina.

Questo metodo si basa sulla formazione di un complesso proteina-rame nel momento in cui viene aggiunta al campione da esaminare il reattivo specifico contenente rame.

Sul complesso proteina-rame agisce il reattivo di Folin, contenente fosfomolibdato che a pH 10 si dissocia in fosfato + molibdato portando ad una riduzione della colorazione gialla, caratteristica del fosfomolibdato, a favore di una colorazione più o meno azzurra a seconda della quantità di proteine presenti nel campione in esame.

I campioni così colorati vengono sottoposti a lettura spettrofotometrica alla lunghezza d'onda di 750 nm e dai valori di assorbanza è possibile risalire ai valori di concentrazione utilizzando la curva standard.

2.8 Immunoblotting

La proteine Bax, STAT3, TSHr, Caveolina ,SMasi ed SMsintasi sono state valutate con l'analisi Western Blot. Circa 30 ug di proteine sono state caricate su elettroforesi in gel di poliacrilammide SDS-PAGE 10% per STAT3 TSHr e Bax, al 12% per Caveolina SMasi e SMsintasi e il trasferimento è avvenuto su lastra di nitrocellulosa in 75 min (*Albi et al*.,2008). Le membrane sono state bloccate per 30 minuti con 0,5% di latte secco senza grassi in PBS, pH 7,5, e incubate tutta la notte a 4 °C con anticorpi specifici. Le membrane sono state trattate con anticorpi secondari coniugati per 90 min.. La visualizzazione è stata effettuata con il kit per la chemiluminescenza potenziato. Le bande immunoblot sono state quantificate con programma Scion Immagine.

2.9 Saggio enzimatico per la valutazione dell'attività della sfingomielinasi

(*Albi and Viola-Magni*, 1997)

L'attività enzimatica della SMasi è stata valutata utilizzando come substrato ^{14}C-SM (attività specifica finale: 1.08 Ci/mol), marcata sul gruppo fosforil-colinico (PPC), incubando il campione ad una concentrazione di 100 ug di proteine. L'incubazione è stata effettuata a 37 °C per 45 min. ed è stata bloccata con 20 v di cloroformio-metanolo 2:1.

Dopo estrazione lipidica il campione viene centrifugato a 2000g per 10 min. e il PPC marcato liberato dalla SMasi viene valutato nella fase inorganica, ponendo il campione in counting vials con 10 ml di Ecoscint e 1 ml di H_2O e valutando la radioattività allo scintillatore Packard.

2.10 Saggio enzimatico per la valutazione dell'attività della sfingomielina sintasi

(*Albi e Viola-Magni, 1999a*)

L'attività enzimatica della SMsintasi è stata valutata utilizzando come substrato il ^{3}H-PC (attività specifica finale: 1.27 μCi/mol), marcata sul gruppo fosforil-colinico incubando il campione ad una concentrazione di 100 ug di proteine. L'incubazione è stata effettuata a 37 °C per 45 min. ed è stata bloccata con 20 v di cloroformio-metanolo 2:1.

Dopo estrazione lipidica il campione è stato centrifugato a 2000g per 10 min. La fase organica è stata evaporata ed è stata aggiunta SM fredda. Sull'estratto è stata effettuata cromatografia TLC, la banda è stata individuata con vapori di iodio e la posizione della SM è stata valutata con lo standard di riferimento. La banda cromatografica è stata raccolta ed è stata posta in counting vials con 10 ml di Ecoscint e 1 ml di H_2O; la radioattività è stata valutata allo scintillatore Packard.

2.11 Dosaggio cAMP

I lobi della tiroide sono stati divisi in 3 frammenti: due frammenti sono stati trattati con 10^{-7} e 10^{-8} M di TSH per 1 ora, il frammento non trattato è stato utilizzato come controllo. Dopo la stimolazione con

TSH i frammenti sono stati fissati con etanolo assoluto per 10 min a temperatura ambiente e centrifugati per 20 min a 3000g.

I sovranatanti sono stati utilizzati per la valutazione dei livelli di cAMP.; a tal fine è stato usato un Kit di dosaggio per cAMP EIA che consente misurazioni di cAMP all'interno di una curva standard da 0,08 a 10 pmol / ml. Le piastre da 96 pozzetti sono state fornite nel kit.

Ogni campione è stato analizzato in doppio a due diluizioni diverse per tre volte; sono stati aggiunti a ciascun pozzetto cinquanta microlitri di standard o del campione. Negli step successivi, eseguiti secondo le istruzioni di produzione, è stato aggiunto prima cAMP acetilcolinesterasi (AChE), che è in grado di legare anticorpi specifici in modo inversamente proporzionale al cAMP libero da dosare nel campione, poi è stato aggiunto il reagente di Ellman contenente il substrato per AChE. Il prodotto della reazione è apparso giallo e l'intensità del colore è stata misurata spettrofotometricamente a 412 nm.

2.12 Estrazione dei lipidi per UFLC-MS/MS

L’estrazione dei lipidi è stata eseguita secondo Matyash et al. 2008, i campioni sono stati diluiti con 1 ml di metanolo, sono stati aggiunti 3 ml di acqua ultra pura e 3 ml MTBE. Ciascun campione è stato vortexato per 1 min e centrifugato a 3000g per 5 min. Il sovranatante è stato recuperato. L'estrazione con MTBE è stata ripetuta ed il sovranatante è stato aggiunto al primo. La fase organica è stata portata a secco sotto flusso di azoto e poi risospesa in 500 ul di metanolo.

2.13 Analisi in liquido-cromatografia associata a spettrometria di massa (UFLC-MS/MS)

Le soluzioni degli standard (SM 18: 1 12: 0, SM 18: 1 16: 0, SM 18: 1 SM 18: 1, ceramide 18: 1 16: 0, ceramide 18: 1 18: 0, ceramide 18: 1 20: 0, ceramide 18: 1 24: 0; sfinganina 18: 1; glucosyl ceramide 18: 1 16: 0 e CHO) sono stati preparati secondo Matyash et al.(2008); gli standard sono stati sospesi in cloroformio / metanolo (9: 1 v/v) a concentrazione finale di 10 ug/ml.

Le soluzioni madre sono state conservate a -20 °C, La curva di calibrazione è stata preparata diluendo in metanolo le soluzioni stock a 500, 250, 100, 50 ng / ml. 20 ul di campione sono stati iniettati dopo purificazione con filtri in nylon specifici (0,2 micron).

Le analisi sono state eseguite secondo *Rabagny et al*., utilizzando un sistema di liquido cromatografia associata ad uno spettrometro di massa Applaied biosistem (Shimadzu Italia srl, Milano, Italia). Le specie lipidiche sono state separate, identificate e analizzate seguendo i metodi di Rabagny et al. (2011).

III. RISULTATI

1- INTRODUZIONE AL I° LAVORO

È noto che nell'ambiente spaziale le cellule tiroidee in coltura subiscono una modificazione strutturale e funzionale per cui si ha un rallentamento della crescita cellulare, in quanto, le cellule non rispondono al trattamento con TSH. È stato precedentemente dimostrato *in vivo* che la mancata risposta al TSH era dovuta ad una perdita del TSHr accanto a modificazioni strutturali della membrana citoplasmatica del tireocita.

L'obiettivo del presente studio è stato quello di valutare le possibili modificazioni della ghiandola tiroidea *in vivo*, partecipando ad esperimenti di "Tissue Sharing" coordinati dal Dott. Cancedda, durante la prima missione spaziale di permanenza per 91 giorni presso l'ISS, in cui i topi venivano mantenuti nella Facility MDS sviluppata dall'ASI, come riportato in materiale e metodi.

A tale scopo sono stati utilizzati sia topi WT e sia topi over-esprimenti pleiotropina (PTN-TG), proteina coinvolta nel metabolismo osseo, al fine di valutare il possibile coinvolgimento della calcitonina tiroidea sulle alterazioni ossee riscontrate negli astronauti al rientro dalle missioni spaziali.

INTRATHYROID REARRANGEMENT IN SPACE ENVIRONMENT

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Accumulating evidence show that in normal thyroid gland there is an intrathyroid regulation among follicular and parafollicular cells. We have previously demonstrated that during space missions, follicular cells in culture released microdomains of membrane constituted by cholesterol and sphingomyelin containing TSHreceptor, thus inducing impairment of TSH-TSHR interaction and consequently lack of response to TSH treatment resulting in the release of low cAMP levels. To study whether the molecular changes of follicular cells could be present also *in vivo,* influencing parafollicular cells and/or vice-versa, we took the opportunity of shifting to *in vivo* research, by joining the "Tissue Sharing" experiment headed by Dr. R. Cancedda, thus participating to the Space Shuttle/ISS 90 days mission inside the Mice Drawer System (MDS) facility developed by the Italian Space Agency. In this longest-duration animal experiment in the history of Space exploration, we have investigated the possible changes of thyroid tissue structure on histological microsections of wild-type (WT) and Pleiotrophin transgenic (PTN-TG) mice stainied with hematoxylin-eosin whereas immunohistochemical analysis was used to highlight calcitonin production. Control animals were maintained in the same conditions in the vivarium of Genoa, Italy. Results showed that WT spaceflight mice presented a more homogenous thyroid tissue structure with ordered follicles, a reduction of interfollicular space with evident loss of parafollicular cells and strong reduction of immunopositivity for calcitonin compared with thyroid gland of control mice. The overexpression of PTN in spaceflight animals did not change the characteristics of follicles present in thyroid gland of PTN-TG maintained in the vivarium but reduced strongly the loss of parafollicular cells observed in WT mice thyroid.

In conclusion, we suppose that modifications of follicular cells during space mission, regulated in turn by hypothalamus, are responsible for parafollicular cell changes, possibly playing a key role in osteoporotic damages observed in astronauts after long-term space missions.

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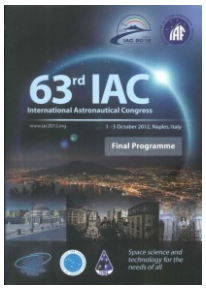
COMMENTO DEL I° LAVORO

I risultati hanno evidenziato che i topi WT presentavano una struttura tiroidea più omogenea accanto ad una riduzione dello spazio interfollicolare rispetto ai topi controllo a terra, mentre i topi PTN-TG avevano una struttura simile ai controlli.

2- INTRODUZIONE AL II° LAVORO

Considerate le modificazioni follicolari e parafollicolari della ghiandola tiroidea abbiamo voluto valutare se ci potessero essere variazioni di molecole coinvolte nella trasformazione tumorale della tiroide durante la missione spaziale. A tale scopo sono stati analizzati HBME-1, MIB-1, CK-19 e galactina-3 come markers tumorali in tiroidi di topo, mantenuti in MDS per 91 giorni in ISS, con tecniche di immunoistochimica.



Galectin-3 leaves thyroid follicular cells during space missions

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Galectin-3 (Gal-3) is the only member of galectin family that exhibits a pentameric structure and thus is capable of crosslinking glycoproteins at the cell surface to form new lattices that are involved in cellular signaling and receptor endocytosis. The N-terminal domain is rich in proline, tyrosine, and glycine residues and enables the formation of pentamers, and thereby plasma membrane galectin lattice microdomains involved in cellular signaling and receptor stabilization (1). Gal-3 is enriched in lipid raft domains of cell membrane (2). Upregulation of Gal-3, and its translocation into the nucleus, occurs in proliferating cells, suggesting a function in normal cell growth (1). We have previously demonstrated an impairment of cell membrane lipid rafts, rich in sphingomyelin (SM) and cholesterol (CHO) content, of follicular cells in culture by participating to several Space missions on board of Sounding rockets, Soyuz and Space Shuttle/International Space Station (ISS), in collaboration with the Sedish Space Corporation, Russian Space Agency, European Space Agency and NASA (3, 4). The consequences were a defect of thyrotropin (TSH) – thyrotropin receptor (TSHR) interaction and a lack of response to TSH treatment resulting in the release of low cAMP levels (3,4). Recently we took the opportunity of shifting to "*In Vivo*" research to study the effect of space environment in thyroid gland in vivo, by joining the "Tissue Sharing" experiment headed by Dr. R. Cancedda, thus participating to the Space Shuttle/ISS 90 days mission inside the Mice Drawer System (MDS) facility developed by the Italian Space Agency. C57BL/10J mice, 8 weeks old at launch, wild type (WT) and osteoblast stimulating factor (OST) transgenic (TG) were used as experimental models. Mice of the same species, sex, and age were housed in MDS for 3 months as the ground controls and mice housed in normal vivarium cage as the laboratory controls. Results showed that spaceflight animals had a more homogenous structure of thyroid tissue compared to the control samples, with a prevalence of ordered and large follicles in which follicular thyrocytes were thicker and the nuclear volumes appeared increased with consequent change of thyroid epithelium vs. colloid volumetric ratio (5). In the intracellular junctions of follicular cells TSHR and caveolin-1 were overexpressed and the response to TSH treatment was higher than control sample (5). The sphingomyelinase moved from the nucleus to the cytoplasm influencing probably the structure/function of the lipid rafts SM (6). In addition the interfollicular spaces were reduced with the loss of C cells and calcitonin production (7). Thus since

the space environment induces in vivo an hyperfunctionality of follicular cells at the expense of those parafollicular, we wanted to evaluate the behavior of Gal-3, normally involved in cell proliferation, in the same experimental model. The results showed that the space environment induces overexpression of Gal-3 that is liberated in the colloid, perhaps by means of lipid rafts. As to work the Gal-3 must translocate into nucleus, it is possible that its leakage in the colloid is a limiting factor for cell proliferation.

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Research Article

How Microgravity Changes Galectin-3 in Thyroid Follicles

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After long-term exposure to real microgravity thyroid gland *in vivo* undergoes specific changes, follicles are made up of larger thyrocytes that produce more cAMP and express more thyrotropin-receptor, caveolin-1, and sphingomyelinase and sphingomyelin-synthase; parafollicular spaces lose C cells with consequent reduction of calcitonin production. Here we studied four immunohistochemical tumor markers (HBME-1, MIB-1, CK19, and Galectin-3) in thyroid of mice housed in the Mouse Drawer System and maintained for 90 days in the International Space Station. Results showed that MIB-1 proliferative index and CK19 are negative whereas HBME-1 and Galectin-3 are overexpressed. The positivity of Galectin-3 deserves attention not only for its expression but also and especially for its localization. Our results highlighted that, in microgravity conditions, Galectin-3 leaves thyrocytes and diffuses in colloid. It is possible that the gravity force contributes to the maintenance of the distribution of the molecules in both basal membrane side and apical membrane side and that the microgravity facilitates slippage of Galectin-3 in colloid probably due to membrane remodelling-microgravity induced.

1. Introduction

Galectins are endogenous lectins which constitute a galactoside-binding protein family of 15 members [1]. All members share close sequence homology in their carbohydrate recognition domain but exhibit different affinities for different saccharide ligands and can be bi- or multivalent in terms of their ligand-binding activity in cell surface [2]. Eukaryotic cell surfaces are dominated by the glycocalyx, a ~100 nm wide macromolecular structure consisting of glycans attached to proteins and lipids and N-glycans appear to be the major ligand for galectins [3]. Each member of the galectin family contains at least one domain of about 130 amino acids; this domain binds to saccharides and is designated the carbohydrate recognition domain (CRD). Based on the number and organization of domains in the polypeptides, the galectins have been classified into subfamilies: (a) the prototype group contains one domain, the CRD; (b) the chimera group contains a proline- (P-) and glycine- (G-) rich domain (also about 130 amino acids) which fused amino terminal to the CRD; and (c) the tandem repeat group contains two CRDs [4].

Galectin-3 (Gal-3), the only representative of the chimera group, was first discovered as an IgE-binding protein and characterized as a 32 kDa antigen on the surface of murine macrophages [5]. It is mainly a cytosolic protein but can easily traverse the intracellular and plasma membranes to translocate into the nucleus or mitochondria or get externalized [6]. The protein shuttles between the cytoplasm and nucleus on the basis of targeting signals that are recognized by importins for nuclear localization and exportin-1 for nuclear export. Depending on the cell type, specific experimental conditions *in vitro*, or tissue location, Gal-3 has been reported to be exclusively cytoplasmic, predominantly nuclear, or distributed between the two compartments [7]. The presence of Gal-3 in the nucleus is dependent on the integrity

of ribonucleoprotein complexes [8] and a Gal-3-U1 small nuclear ribonucleoprotein (snRNP) complex has been identified, which provides a mechanism of incorporation of the Gal-3 into the pre-mRNA splicing substrate [9]. In addition, Gal-3 is secreted via nonclassical pathway outside of the cell independent on the classical secretory pathway through the endoplasmic reticulum/Golgi network thus being found on the cell surface or in the extracellular space [10]. Thus, Gal-3 is a multifunctional protein, which regulates pleiotropic biological functions such as cell growth, cell adhesion, cell-cell interactions, apoptosis, angiogenesis, and mRNA processing. Its unique structure enables interacting with a plethora of ligands in a carbohydrate dependent or independent manner [6].

In thyroid gland, Gal-3 plays a role in the pathogenesis of well-differentiated carcinoma, particularly in papillary carcinoma [11]. Therefore, it is one of the markers most commonly used to assist in distinguishing thyroid lesions together to human bone marrow endothelial cell-1 (HBME-1) as a tumor marker of follicular origin and cytokeratin-19 (CK-19) with general intense and diffuse expression in papillary carcinoma and heterogeneous labeling in carcinoma and in follicular adenoma [12]. In addition MIB-1 is useful in evaluating proliferative activity and in predicting the aggressiveness of thyroid carcinoma [13].

We have previously demonstrated that microgravity induces changes in the physiology of the thyroid gland. In fact, in comparison with control animals, thyroids of spaceflight animals have a more homogenous structure, produce more cAMP, and overexpress thyrotropin-receptor (TSHR), caveolin-1 [14], and sphingomyelinase and sphingomyelin-synthase [15] and are characterized by a loss of parafollicular cells with reduction of calcitonin production [16].

Data are not available at the time regarding the evaluation of thyroid tumor markers in microgravity. We report for the first time the effect of long-term exposure to real microgravity environment on thyroid HBME-1, MIB-1, CK19, and Gal-3.

2. Materials and Methods

2.1. Experimental Design and Animal Care. All experimental procedures were authorized by the Public Veterinary Health Department of the Italian Ministry of Health. The experiment was also conducted in accordance with the regulations for the care and use of laboratory animals and with the guidelines of the Japanese Physiological Society. Furthermore, this study was also approved by the Committee on Animal Care and Use at Graduate School of Medicine, Osaka University (no. 22-071). Finally, the protocol utilized in the study has been authorized by the Public Veterinary Health Department of the Italian Ministry of Health. All experiments were carried out using male C57BL/10J mice (8 weeks old).

2.2. Microgravity Experiment. 3 mice were individually housed in the Mouse Drawer System (MDS), a 11.6 × 9.8 × 8.4 cm payload developed by Thales-Alenia Space Italy and all treatments were performed as previously reported [14]. Food and water were supplied *ad libitum*. The MDS was launched in the Space Shuttle Discovery, within the Space Transport System (STS)-128 mission, on August 28, 2009. It was then housed in Japanese Experimental Module (Kibou) on the ISS until its return to the Earth by Space Shuttle Atlantis (STS-129 mission) on November 27, 2009. Only 1 mouse returned to the Earth alive after 91 days of space flight.

Thyroids were sampled bilaterally from each mouse killed by inhalation of carbon dioxide at the Life Sciences Support Facility of Kennedy Space Center within 3-4 hours after landing and either processed or frozen immediately, according to the various experimental protocols. The procedure was approved by the IACUC protocol n° FLT-09-070(KSC).

After the spaceflight experiment, the on-ground experiment was also carried out at the Vivarium of the Advanced Biotechnology Center in Genoa, Italy. One group of 3 mice with the same species, sex, and age was housed in normal vivarium cage as the laboratory control. Amount of food and water supplementation and environmental conditions were simulated as the flight group. After 3 months, thyroids were sampled bilaterally and treated for spaceflight mice.

2.3. Thyroid Tissue Treatment. The thyroid lobes were fixed in 4% neutral phosphate-buffered formaldehyde solution for 24 h as previously reported [14]. Thyroids were dropped with essentially random orientation in paraffin. The paraffin blocks were sectioned into 4-μm-thick sections. All sections were mounted on silane-coated glass slides. Each slide contained a pair of sections at a distance equal to 140 μm. Between 5 and 14 pairs of sections were sampled excluding the first and the last; sections 2, 6, and 10 were used for HBME-1 detection, sections 3, 7, and 11 for MIB-1 detection, sections 4, 8, and 12 for CK19 detection, and sections 5, 9, and 13 for Gal-3 detection. Tissue sections were deparaffinized and rehydrated through a series of xylene and ethanol washes.

2.4. Immunohistochemical Analysis. For immunohistochemical analysis Bond Dewax solution was used for removal of paraffin from tissue sections before rehydration and immunostaining on the Bond automated system (Leica Biosystems Newcastle Ltd, UK) as previously reported [17]. Immunostaining detection was performed according to Bancroft and Stevens [18] by using HBME-1 and Ki-67 (MIB-1 clone) from Dako (Milano, Italy) and CK19 and Gal-3 antibodies and Bond Polymer Refine Detection from Leica Biosystems (Newcastle Ltd, UK). The observations were performed by using inverted microscopy EUROMEX FE 2935 (ED Amhem, The Netherlands) equipped with a CMEX 5000 camera system (40x magnification). The analysis of the tissue section size was performed by ImageFocus software.

2.5. Statistical Analysis. The experiments have been conducted on the thyroid of 1 animal for the microgravity experiment (the only ones that returned alive from the mission) and 3 control animals for the microgravity experiment (vivarium 1). Median and range of sections 2, 6, and 10 (HBME-1), of sections 3, 7, and 11 (MIB-1), of sections 4, 8, and 12 (CK19), and of sections 5, 9, and 13 (Gal-3) were given.

3. Results and Discussion

Prolonged space flights are known to elicit changes in human cardiovascular, musculoskeletal, immune, and nervous systems whose functions are regulated by the thyroid gland [14]. The structure of thyroid shows the presence of follicles, containing colloid and surrounded by a single layer of thyroid epithelial cells or thyrocytes that produce the metabolically active iodothyronines, and parafollicular spaces with thyroid C cells that produce calcitonin [19]. We have previously reported that thyrocyte cells in culture delay cell growth and enter into a proapoptotic state after long stay on the International Space Station (ISS) [20]. *In vivo* experiments on the board of ISS showed that thyroid of spaceflight mice has more ordered follicles with thicker thyrocytes containing increased nuclear volume [14] and reduction of interfollicular space with loss of C cells [16] in comparison with thyroid gland of ground mice. In order to verify whether the structural changes of the thyroid gland in microgravity conditions could lead to pathological conditions, in this study we investigated the immunoexpression of markers known to be related to clinical outcome. The limitation of the present paper is that only 1 mouse survived to the 91-day spaceflight. However the MDS experiment was a unique opportunity to study the microgravity long-term exposure effects on several tissues of an animal model and to collect interesting observations that could prepare the field to future experiments. The results showed that microgravity gives a nonspecific staining in the colloid during MIB-1, CK19, and Gal-3 immunohistochemistry analysis, absent in control samples. It is really hard to pinpoint the reason but it is possible to hypothesize an increase of membrane permeability microgravity-dependent on the basis of the observation that, at the end of the spaceflight, endothelial cells display profound changes indicating cytoskeletal lesions and increased cell membrane permeability [21]. MIB-1 and CK19 immunopositivity do not show changes in thyroid of spaceflight mice in comparison with control animals (Figure 1(a)). Differently, the immunostaining is present for HBME-1 and it is very strong for Gal-3 (Figure 1(a)). Alshenawy demonstrated that no single marker is completely sensitive and specific for diagnosis of thyroid lesions but only their combination [22] with Gal-3 + HBME-1 was considered the best combination for distinguishing benign from malignant lesions [23]. In thyroid of spaceflight mice the structure of thyroid follicles is more organized than that of the control animals [14] and thyrocytes delay their growth [20] and MIB-1 is negative. So it is very difficult at the moment to consider that the expression of HBME-1 and Gal-3 markers is linked to tumor transformation. However, the possibility that HBME-1 and Gal-3 overexpression might indicate a premaligne state of thyroid tissue cannot be excluded by considering that in microgravity follicles are made up of cells 2 times larger and colloid darker [14] similar to those of papillary carcinoma [24]. Our result showed that HBME-1 is present only in trace in thyroid of control mice maintained in the vivarium whereas it appears evident after space flight with well-defined localization in thyrocytes (Figure 1(a)). Median and range value of immunopositive surface area is 4,62 (5,51–4,57) mm^2, and its ratio in relation

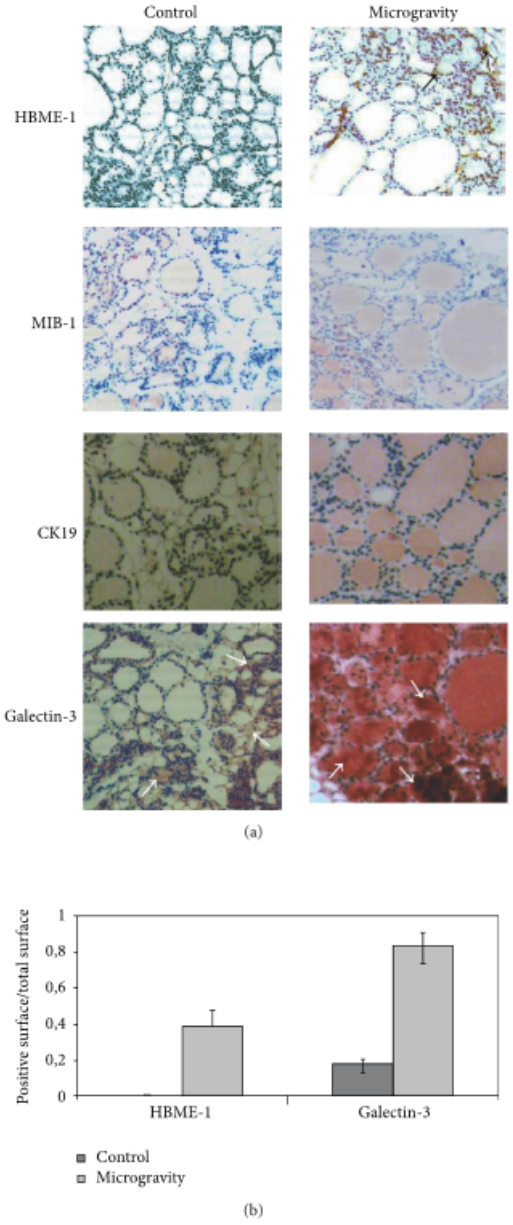

Figure 1: Effect of microgravity on HBME-1, MIB-1, CK19, and Galectin-3. (a) Marker detection in thyroid tissue by immunohistochemical staining. "Control," mice maintained in vivarium cages; "microgravity," experimental mice in space environment. (b) Ratio between the immunopositive surface and total surface of thyroid lobe. The values are expressed as median and range of two sections as reported in Material and Methods, 40x magnification. The arrows indicate positive areas.

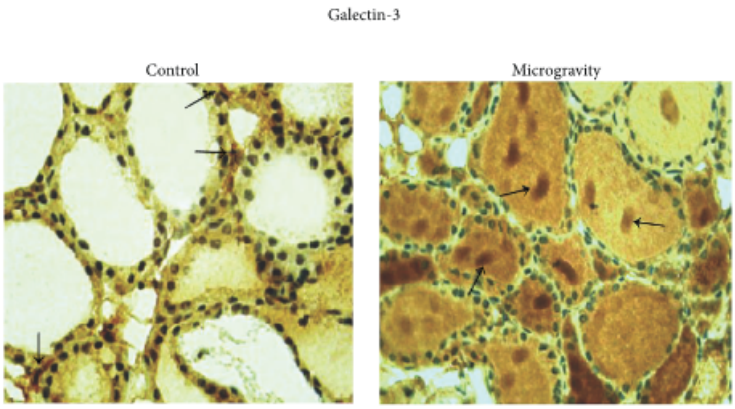

FIGURE 2: Localization of Galectin-3 in colloid. Gal-3 immunohistochemical staining. "Control," mice maintained in vivarium cages; "microgravity," experimental mice in space environment, 40x magnification. The arrows indicate positive areas.

to total surface is reported in Figure 1(b). Gal-3 labelling is present in some of follicular thyrocytes of control animals and it increases strongly in spaceflight mice (Figure 1(a)). Median and range value of immunopositive surface area is 1,72 (1,99–1,25) mm^2 in the control and 7,94 (8,59–7,00) in microgravity by increasing 4,67 times the positive surface/total surface ratio (Figure 1(b)). The presence of Gal-3 in normal thyroid tissue has already been demonstrated [25]. Our data show an overexpression in microgravity. We do not have support in the literature since this is the first study on observation of the behavior of thyroid pathological markers in microgravity. Nevertheless Grosse et al. demonstrated that NF-κB is overexpressed and different factors that interact with it are differentially regulated under altered gravity conditions [26]. In addition spaceflight conditions change gene expression profile in thyroid cancer cells [27]. Therefore microgravity influences gene expression and consequently protein content. However, the positivity of Gal-3 deserves attention not only for its expression but also and especially for its localization. Our results highlighted that, in microgravity conditions, Gal-3 leaves thyrocytes and diffuses in colloid (Figure 2). It is possible that microgravity induces changes of cell membrane that in turn facilitates the escape of Gal-3 accumulated in thyrocytes. We have previously demonstrated that thyrocytes in culture (FTRL-5 cell line) release thyrotropin receptor, linked to cholesterol and sphingomyelin, in culture medium during space missions by indicating a depletion of lipid rafts and consequently cell membrane remodelling [20]. Clarke et al. told about microgravity-induced decrease in membrane order [28] and Hsu et al. localized Gal-3 in membrane lipid rafts [29]. It is possible to suppose that Gal-3 overexpressed in thyrocytes moves into colloid due to the modification of the cell membrane following the variation of gravity force. It has been demonstrated that Gal-3 is mainly a cytosolic protein but it shuttles to the nucleus or extracellular space the basis of targeting signals [6]. Here we do not have specific staining in these locations but the molecules move in the opposite direction; they do not protrude from the basal membrane of thyrocytes towards the extracellular space but from the apical membrane to the colloid. On the other hand, Delacour et al. suggested a direct role of Gal-3 in apical sorting as a sorting receptor [30]. It is possible that the gravity force contributes to the maintenance of the distribution of the molecules in both basal membrane side and apical membrane side and that the microgravity facilitates slippage of Gal-3 in colloid.

4. Conclusion

To our knowledge this is the first study correlating thyroid tumor markers with long stay mice in microgravity conditions. Here we found higher expression of HBME-1 and Gal-3 in comparison with ground gravity. However MIB-1 proliferative index and CK19 are negative. Gal-3, usually present in cytoplasm, nuclei, and extracellular space, leaves thyrocytes and diffuses in colloid probably due to membrane remodelling-microgravity induced.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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COMMENTO DEL II° LAVORO

I risultati hanno mostrato che in ambiente spaziale l'indice proliferativo MIB-1 e CK-19 risultano essere negativi mentre HBME-1 e Galectina-3 sono over-espressi nella tiroide di topo.

Particolare importanza è il comportamento della galectina, non solo per la sua over-espressione ma particolarmente per la sua localizzazione. Infatti i risultati hanno messo in luce che, in condizioni di microgravità la Galectina-3 abbandona i tireociti e diffonde nella colloide.

3- INTRODUZIONE AL III° LAVORO

A questo punto era chiaro che la ghiandola tiroidea subiva modificazioni strutturali e funzionali ma era dunque importante valutare i possibili mecchanismi molecolari che ne erano alla base. Poiché era noto dalla letteratura recente che il TSHr è localizzato in microdomini lipidici ricchi di SM e CHO, la nostra attenzione è stata rivolta al comportamento del metabolismo della SM in topi inviati per 91 giorni in ISS.

Al fine di valutare se gli eventuali effetti riscontrati fossero legati alla microgravità di per se stessa, oppure se una qualsiasi variazione di forza di gravità potesse indurre un simile modificazione, l'esperimento è stato condotto anche in condizione di Ipergravità.

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Research Article

Observing the Mouse Thyroid Sphingomyelin Under Space Conditions: A Case Study from the MDS Mission in Comparison with Hypergravity Conditions

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Abstract

This is a case report of apparent thyroid structural and functional alteration in a single mouse subjected to low Earth orbit spaceflight for 91 days. Histological examination of the thyroid gland revealed an increase in the average follicle size compared to that of three control animals and three animals exposed to hypergravity (2*g*) conditions. Immunoblotting analysis detected an increase in two thyroid gland enzymes, sphingomyelinase and sphingomyelin-synthase1. In addition, sphingomyelinase, an enzyme confined to the cell nucleus in the control animals, was found in the mouse exposed to hypogravity to be homogeneously distributed throughout the cell bodies. It represents the first animal observation of the influence of weightlessness on sphingomyelin metabolism. Key Words: Gravity—Lipid—Spaceflight—Sphingomyelin—Thyroid. Astrobiology 12, xxx–xxx.

1. Introduction

Perturbations of body homeostasis in astronauts largely depend on the deregulation of the endocrine system with consequent hormonal disequilibrium. Unfortunately, the underlying mechanisms are still unknown. We have recently demonstrated that, during a space mission, thyroid cells in culture (FRTL-5 cells) did not respond to thyrotropin (TSH) treatment because of the change of sphingomyelin (SM) metabolism mediated by sphingomyelinase (SMase) and SM-synthase enzymes (Albi *et al.,* 2010) and acquired a pro-apoptotic phenotype, so called because it had intermediate characteristics between the quiescent and apoptotic phenotype (Albi *et al.,* 2008). In the cell membrane, SM interacts with cholesterol, phosphatidylcholine (PC), and proteins to form specific microdomains, called "lipid rafts," that act as platforms for specific proteins involved in signal transduction (Edidin, 2003). The presence of TSH receptors (TSHRs) and their multimerization in lipid rafts have been demonstrated (Latif *et al.,* 2003, 2007). Our experiments performed under microgravity conditions (Texus-44 mission) showed that cells released cholesterol, SM, and TSHR to the culture medium probably by modifying the microdomain structure (Albi *et al.,* 2011). The values of cyclic adenosine monophosphate (cAMP) after treatment with TSH were significantly lower than those obtained in Earth's gravity because of TSHR reduction and defect of TSH-TSHR interaction (Albi *et al.,* 2011). So far, experiments were conducted on thyroid cells in culture. To study whether these effects were present *in vivo* in the thyroid gland, which influences cardiovascular, musculoskeletal, and nervous system function through hormone production, we participated in the Mouse Drawer System (MDS) Tissue Sharing Program by performing experiments on mice maintained on board the International Space Station (ISS) during the long-duration (90 days) exploration mission STS-129. The results were compared with those obtained in hypergravity conditions on mice maintained in a 2*g* centrifuge. The study allowed us to asses the behavior of SM metabolism enzymes and Bax *in vivo* after the change of gravity.

2. Materials and Methods

2.1. Reagent

Anti-SMase, anti-SM-synthase, and anti-Bax primary antibodies, horseradish peroxidase–conjugated, tetramethyl rhodamine isothiocyanate (TRITC)–conjugated, and fluorescein isothiocyanate (FITC)–conjugated secondary antibody were obtained from Santa Cruz Biotechnology, Inc. (CA, USA). SDS-PAGE molecular weight standards were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Chemiluminescence kits were purchased from Amersham (Rainham, Essex, UK). Thin layer chromatography plates (silica Gel G60) were from Merck (Darmstadt, Germany); the radioactive SM (choline-methyl ^{14}C, 54.5 Ci/mol), PC (L-3-

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phosphatidyl *N*-methyl-^{3}H choline 1,2 dipalmitoyl, 81.0 Ci/mmol) were from Amersham Pharmacia Biotech (Rainham, Essex, UK); Ecoscint A was from National Diagnostic (Atlanta, GA, USA).

2.2. Experimental design and animal care

All experimental procedures were authorized by the Public Veterinary Health Department of the Italian Ministry of Health. The experiment was also conducted in accordance with regulations for the care and use of laboratory animals and with the guidelines of the Japanese Physiological Society. Furthermore, this study was also approved by the Committee on Animal Care and Use at Graduate School of Medicine, Osaka University (No. 22-071). Finally, the protocol utilized in the study has been authorized by the Public Veterinary Health Department of the Italian Ministry of Health. All experiments were carried out with male C57BL/10J mice (8 weeks old).

Hypogravity experiment. Three male C57BL/10J mice were individually housed in the MDS, a 11.6×9.8×8.4 cm payload developed by Thales-Alenia Space Italy (Cancedda *et al.*, 2002). Food and water were supplied *ad libitum*. The MDS was launched on the Space Shuttle Discovery, within the Space Transport System (STS)-128 mission, on August 28, 2009. It was then housed in the Japanese Experimental Module (Kibou) on the ISS until its return to Earth via Space Shuttle Atlantis (STS-129 mission) on November 27, 2009 (Masini *et al.*, 2012). Only one mouse returned to the Earth alive after 91 days of spaceflight. It was very difficult to establish the cause of death because this was the first long-term animal experimentation in space. It was not possible to perform an autopsy on the two dead animals because they were not frozen immediately after death.

After the spaceflight experiment, the on-ground experiment was also carried out at the vivarium of the Advanced Biotechnology Center in Genova, Italy. One group of mice with the same species, sex, and age were housed in a normal vivarium cage as the laboratory control (vivarium 1). The amount of food and water supplementation and the environmental conditions were simulated for the control group to approximate those of the flight group. All analyses were conducted on the thyroid gland of one animal alive. Thyroids were sampled bilaterally from each mouse killed by inhalation of carbon dioxide at the Life Sciences Support Facility of Kennedy Space Center within 3–4 hours after landing. The thyroid samples were either processed or frozen immediately, according to the various experimental protocols.

Hypergravity experiment. Three mice of the same strain as those used in hypogravity experiments were maintained in hypergravity, with conditions similar to the MDS experiment, in a 2*g* centrifuge in the laboratory of Dr. Y. Ohira at the Osaka University, Osaka, Japan. Control mice were similar to those reported in the hypogravity experiment (vivarium 2). Animals were treated, and thyroids were obtained and processed with the same procedures used in the hypogravity/space experiments.

2.3. Thyroid tissue treatment

The thyroid lobes in part were homogenized and used for protein dosage, immunoblotting analysis, and enzyme activity assay, and in part fixed in 4% neutral phosphate-buffered formaldehyde solution for 24 h. Thyroids were dropped with essentially random orientation in paraffin. The paraffin blocks were sectioned into 4 μm thick sections. All sections were mounted on silan-coated glass slides. Each slide contained a pair of sections at a distance equal to 140 μm. Between 7 and 14 pairs of sections were sampled, excluding the first and the last, and 7, 9, 11, and 13 sections were used for morphological analysis whereas 8, 10, 12, and 14 sections were used for immunofluorescence analysis. Tissue sections were deparaffinized and rehydrated through a series of xylene and ethanol washes.

2.4. Morphological analysis

The sections were treated with the hematoxylin-eosin (Chroma-Gesellschaft, Germany) staining method and investigated by using inverted microscopy EUROMEX FE 2935 (ED Amhem, the Netherlands) equipped with a CMEX 5000 camera system (40× magnification). The analysis of the tissue section size was performed by ImageFocus software.

2.5. Protein study

The proteins were quantified according to Lowry *et al.* (1951). The immunoblotting analysis to detect SMase, SM-synthase, and Bax content was performed as previously reported (Albi *et al.*, 2011). Enzyme activity of SMase and SM-synthase1 was assayed according to Albi *et al.* (2008). For immunofluorescence analysis, sections were incubated, after three washes with phosphate-buffered saline (PBS), with 2 μg/mL anti-SMase or anti-SM-synthase1 primary antibodies diluted in a 0.5% solution of bovine serum albumin in PBS overnight at 4°C. The slides were washed three times with PBS and incubated with fluorochrome-conjugated secondary antibodies (TRITC for SMase and FITC for SM-synthase), diluted as primary antibodies, for 1 h at room temperature. After three washes with PBS, the slides were mounted with glycerol and coverslips. The samples were examined under a fluorescence microscope (OLYMPUS IX 51) equipped with an OLYMPUS DP 50 camera system and analyzed at 40× magnification.

2.6. Statistical analysis

The experiments were conducted on the thyroid of one animal for the hypogravity experiment (the only one returned alive from the mission), three control animals for the hypogravity experiment (vivarium 1), three animals for the hypergravity experiment, three control animals for the hypergravity experiment (vivarium 2). The immunoblotting analysis and enzyme activity assay were repeated three times on each thyroid. Means ± SD of three results for hypogravity animals and of nine results for each other experiment are given. The significance of the differences between the data was checked by the Student *t* test.

3. Results

Microscopy analysis performed on four histological microsections, as reported in "thyroid tissue treatment," which were subjected to hematoxylin-eosin staining, showed that the thyroid gland of the animal that had been in space has a greater number of large follicles, which supports recent

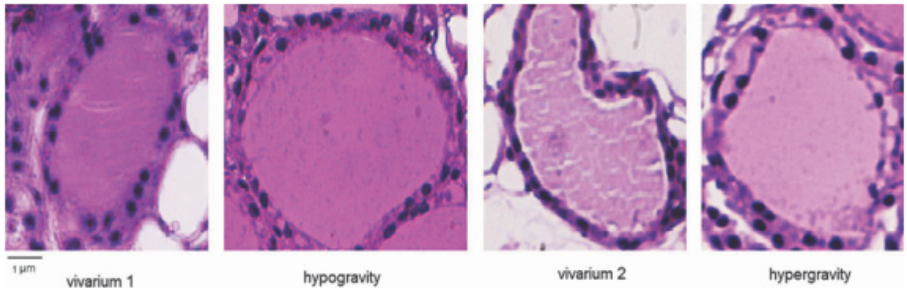

FIG. 1. Morphology analysis of thyroid follicles of mice. "vivarium 1," maintained in vivarium cages (control for experiment in hypogravity); "hypogravity," experimental animal in space; "vivarium 2," control for experiment in hypergravity; "hypergravity," experimental animals in 2*g* centrifuge. Hematoxylin-eosin staining, 40× magnification, 1 μm scale bar. Color images available online at www.liebertonline.com/ast

observations (Masini *et al.*, 2012). The differences of the follicle surface area are shown in Fig. 1 and Table 1.

Experiments of immunoblotting demonstrated that the long-term exposure to a real microgravity environment increased the levels of SMase and SM-synthase1 (Fig. 2a). The band density of SMase, corresponding to 42 kDa apparent molecular weight, was 2.18-fold higher than that of the vivarium 1 control, whereas the hypergravity reduced the enzyme 1.79 fold with respect to the vivarium 2 control (Fig. 2b). Immunofluorescence analysis confirmed these data and highlighted a different distribution of the enzyme with gravity change in comparison with control samples. In fact, in vivarium 1 and vivarium 2 the enzyme appeared particularly localized inside the cells, as specific round images, whereas in hypogravity the enzyme had a more uniform distribution and the hypergravity sample showed a peripherical localization (Fig. 3). The immunoblotting of SM-synthase1 showed a strong immunopositivity of band, corresponding to 49 kDa apparent molecular weight, only in the sample in hypogravity (Fig. 2a). The band area analysis showed an enrichment of about 20-fold in hypogravity in comparison to vivarium 1, whereas no variations were present in hypergravity with respect to its control (Fig. 2b). These data were confirmed with the immunofluorescence technique (Fig. 4).

To analyze whether the SMase and SM-synthase1 in hypogravity and hypergravity were active, the enzyme activities of both enzymes were assayed. Hypogravity resulted in a 5.45- and 17.66-fold increase in SMase and SM-synthase activity, respectively, in comparison to the controls, while hypergravity resulted in a 1.95 and 10.55 increase in SMase and SM-synthase (Fig. 5a). The hypogravity increased 5.45- and 17.66-fold, whereas hypergravity resulted in a 1.95- and 10.55-fold increase in SMase and SM-synthase activity, respectively, in comparison with their controls (Fig. 5a). However, with regard to the enzyme activity in relation to the band density, it was evident that the increase of SMase activity was similar in hypogravity and hypergravity, and no variation was found for SM-synthase1 activity (Fig. 5b).

The band of Bax, corresponding to 23 kDa apparent molecular weight, showed a higher immunopositivity either in hypogravity or in hypergravity with respect to control animals (Fig. 6a). The band area density analysis demonstrated that the value was 2- and 4-fold higher than that observed in vivarium 1 and vivarium 2, respectively; by comparison, the values in hypogravity and hypergravity were very similar (Fig. 6b).

Table 1. Surface Area of Thyroid Follicles

Vivarium 1	14.9 ± 5.7
Hypogravity	35.4 ± 1.9**
Vivarium 2	20.6 ± 3.5
Hypergravity	27.39 ± 2.8

The surface area of large follicles was analyzed by ImageFocus software. Data represent the mean ± SD of three analyses performed in thyroid gland of three mice maintained in vivarium and used as controls for experiment in hypogravity (Vivarium 1), of three analyses performed in thyroid gland of the mouse maintained in hypogravity, of three mice maintained in vivarium and used as controls for experiment in hypergravity (Vivarium 2), and of three mice maintained in hypergravity. Significance: $^{**}P < 0.001$ hypogravity versus vivarium 1.

4. Discussion

Although we were able to discern by way of FRTL-5 thyroid cells in culture the effect of the space environment on the involvement of SM metabolism in the change of cell function (Albi *et al.*, 2010), there have been no reports of this effect in thyroid glands *in vivo*. Our data show clearly that in the control sample the SMase was localized specifically in the nucleus, whereas in hypogravity the enzyme moved from the nucleus and was homogeneously distributed in the cells. Hypogravity induced an overexpression of both enzymes with a diffuse localization of SMase. Apparently, the activity of SM-synthase1 increased more than that of SMase, but considering the activity in relation to the enzyme content, only SMase activity increased. Therefore, only the SMase of mouse thyroid was more active in hypogravity, which is similar to results obtained *in vitro* in FRTL-5 cells. This represents the first animal observation of the influence of weightlessness on SM metabolism. Out of the three animals sent to space inside the MDS on board the Shuttle Discovery and then transferred to the ISS, only one returned to Earth alive after the 91-day space mission. Usually, such a low number of experimental animals would be considered inadequate for conducting scientific analyses. But because

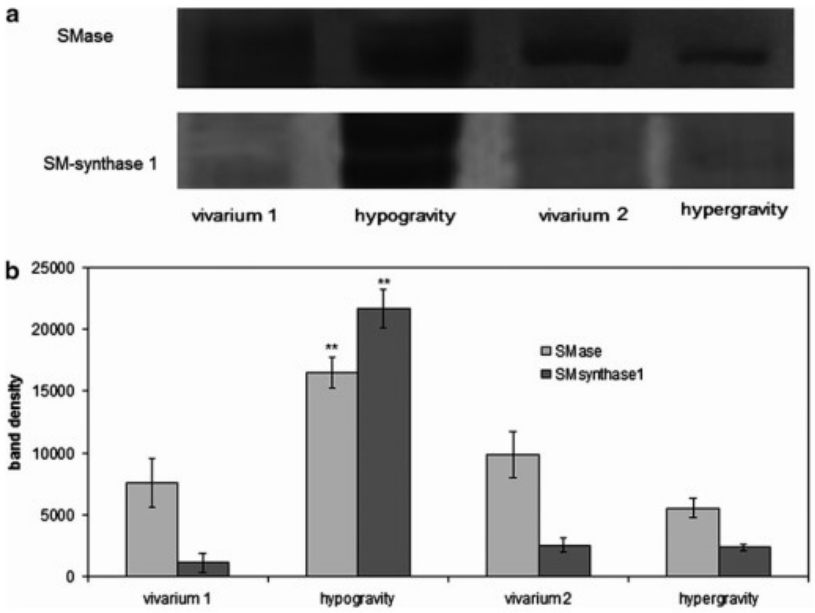

FIG. 2. Comparison of SMase and SM-synthase1 content by immunoblotting analysis. The study was performed by using specific antibodies. The position of the 43 kDa apparent molecular weight for SMase and 49 kDa for SM-synthase1 is indicated comparing the position of molecular size standards. The area density was evaluated by densitometry scanning and analyzed with Scion Image program. Data represent the mean ± SD of three analyses performed in the thyroid gland of three mice maintained in vivarium and used as controls for experiment in hypogravity (vivarium 1), of three analyses performed in the thyroid gland of the mouse maintained in hypogravity, of three mice maintained in vivarium and used as controls for the experiment in hypergravity (vivarium 2), and of three mice maintained in hypergravity. Significance: $^{**}P < 0.001$ hypogravity versus vivarium 1.

of the exceptionality of the experimental substrate (animals kept in space for the longest time, to date) and the improbability that such an experiment will be repeated within a reasonable amount of time, we present the results as a case report. Given that we recently demonstrated that the changes in the thyroid cell membrane during spaceflight are due to weightlessness (Albi *et al.*, 2011), we conjectured that the modifications of SM metabolism enzymes shown in this case could be due to the hypogravity and were therefore interested in a comparison of the results with those

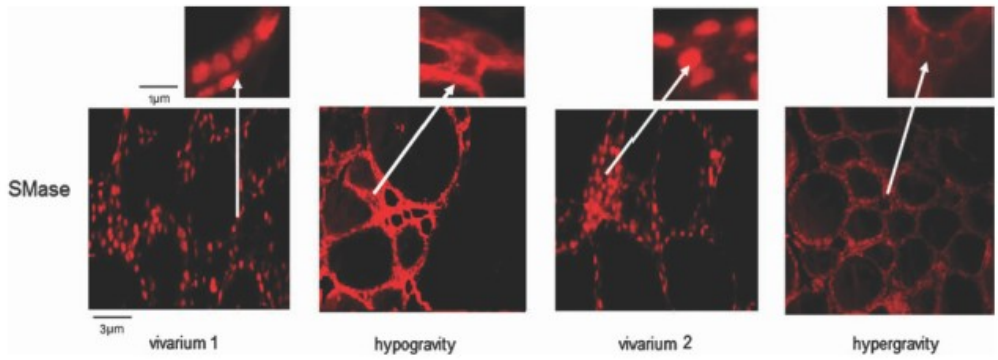

FIG. 3. Fluorescence immunostaining of SMase in thyroid tissues. Analyses were performed using anti-SMase primary antibody and TRITC-conjugated secondary antibody. 40× magnification, 3 μm scale bar. The arrows indicate particulars at 100× magnification, 1 μm scale bar. ("vivarium 1," control for the hypogravity experiment; "vivarium 2," control for the hypergravity experiment.) Color images available online at www.liebertonline.com/ast

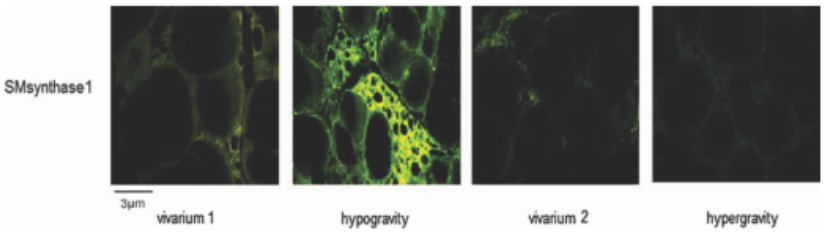

FIG. 4. Fluorescence immunostaining of SM-synthase1 in thyroid tissues. Analyses were performed by using anti-SM-syntase1 primary antibody and FITC-conjugated secondary antibody. 20×magnification. Color images available online at www.liebertonline.com/ast

obtained under hypergravity conditions. It would seem that hypogravity and hypergravity would generate opposite results, but some key results of our study were surprisingly similar. In fact, the most relevant distinctions to be made are that in hypergravity no variation of expression of SMase and SM-synthase1 were found. The SMase translocated, however, from the nucleus to the cytoplasm as occurs in hypogravity; and its activity, calculated in relation to the band density of the enzyme, had similar values. This implies that, although the SMase was present in thyroid with different content in hypogravity and hypergravity, the change of gravity could be responsible for a molecular remodeling that could influence the cell fate. It is possible that the change of gravity induces functional proteins to trigger modifications in cellular response and therefore significantly modifies thyroid function. It is interesting to note that, in analogy to SMase, the level of Bax is similar in hypogravity and hypergravity as well. Thus, as the associated behavior of the two proteins was previously observed when FRTL-5 thyroid cells entered into a pro-apoptotic state at the reentry of a space mission (Albi *et al.*, 2010), we speculate that the cross-talk between the two proteins was stimulated by the change of gravity. On the other hand, morphological analysis of thyroid follicles also revealed large differences in comparison to the control samples but significant similarities between the hypogravity and hypergravity conditions. This supports our hypothesis of the significant influence of gravity on the partitioning of

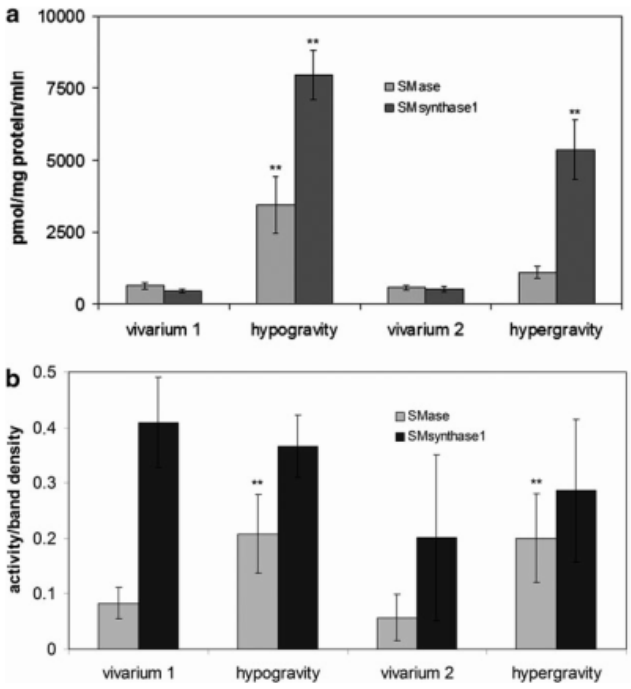

FIG. 5. Activity of SMase and SM-synthase1. The enzyme activity was evaluated as (**a**) cpm/mg protein/min; (**b**) cpm/mg protein/min in relation to band density analyzed in Fig. 2. Data represent the mean±SD of three analyses of one experiment for the hypogravity sample and of three analyses of three independent experiments for the other samples. Significance: $^{**}P<0.001$ hypogravity versus "vivarium 1" (control 1) and "hypergravity" versus "vivarium 2" (control 2).

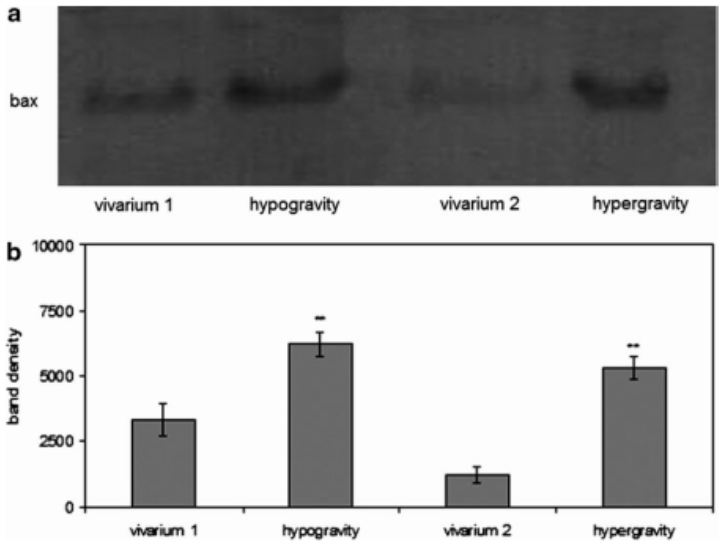

FIG. 6. Comparison of Bax content by immunoblotting analysis. The study was performed by using a specific antibody. The position of the 23 kDa apparent molecular weight was indicated in comparison to the position of molecular size standards. The area density was evaluated by densitometry scanning and analyzed with Scion Image program. Data represent the mean ± SD of three analyses of one experiment for the hypogravity sample and of three analyses of three independent experiments for the other samples. Significance: $^{**}P < 0.001$ hypogravity versus vivarium 1. ("vivarium 1," control for the hypogravity experiment; "vivarium 2," control for the hypergravity experiment.)

relevant molecules within the cell membrane. Looking ahead, the quest will now be to understand the interplay between the lipid environment and protein activities to identify possible countermeasures that are effective in preserving the integrity of the thyroid structure and function.

Acknowledgments

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Glossary

Apoptosis: process of programmed cell death.

Bax: pro-apoptotic Bcl-2 protein.

Cyclic adenosine monophosphate (cAMP): second messenger involved in signal transduction.

Lipid raft: liquid-ordered microdomain, formed by sphingomyelin with a high affinity for cholesterol, in a liquid-disordered phase of cell membrane.

Signal transduction: the process by which an extracellular molecule activates a membrane molecule, that in turn induces a response by acting on intracellular molecules.

Sphingomyelin metabolism: sphingomyelin, a major lipid constituent of cell membranes, important in cell signaling, is degraded by sphingomyelinase and produced by sphingomyelin-synthase.

Thyrotropin: or thyroid-stimulating hormone (TSH) released by the anterior pituitary gland, located at the base of the brain, that regulates the activity of the thyroid gland.

Abbreviations

FITC, fluorescein isothiocyanate; ISS, International Space Station; MDS, Mouse Drawer System; PBS, phosphate-buffered saline; PC, phosphatidylcholine; SM, sphingomyelin; SMase, sphingomyelinase; STS, Space Transport System; TRITC, tetramethyl rhodamine isothiocyanate; TSH, thyrotropin; TSHR, TSH receptor.

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COMMENTO DEL III° LAVORO

I Risultati hanno mostrato chiaramente che in ipogravità vi era un' over-espressione della SMasi, deputata al catabolismo della SM, e della SMsintasi, deputata alla sintesi del SM. Di particolare rilievo è la variazione della localizzazione enzimatica con l'ipo-gravità. Infatti, nei topi controllo la SMasi era localizzata in maniera specifica nel nucleo del tireocita mentre in ipo-gravità l'enzima si trasferiva dal nucleo e si disponeva in maniera omogenea in tutta la cellula. In aggiunta la SMasi risultava essere più attiva in ipogravità rispetto ai topi controllo.

In ipergravità non sono state evidenziate variazioni di espressione di SMasi ed SMsintasi ma, sia la traslocazione nucleo-citoplasma e sia il livello di attività della SMasi era simile a quella ottenuta in Ipogravità.

4- INTRODUZIONE AL IV° LAVORO

Le cellule parafollicolari o cellule C della tiroide sono note per produrre calcitonina, ormone implicato nel metabolismo osseo. Poiché gli astronauti, al rientro dalle missioni spaziali, presentano alterazioni ossee, imputate in letteratura ad un danno diretto della mancanza della forza fisica di gravità sui processi di formazione e riassorbimento osseo, abbiamo voluto valutare un possibile coinvolgimento della calcitonina a tale danno.

Atale scopo sono stati utilizzati topi mantenuti per 91 giorni in MDS sulla ISS sia di tipo WT e sia over-esprimenti pleiotropina (PTN-TG), molecola che produce effetti positivi sul turn-over osseo.

Al fine di valutare l'importanza della forza di gravità è stata studiata la variazione delle cellule parafollicolari e della calcitonina anche in condizioni di ipergravità.

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Loss of Parafollicular Cells during Gravitational Changes (Microgravity, Hypergravity) and the Secret Effect of Pleiotrophin

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Abstract

It is generally known that bone loss is one of the most important complications for astronauts who are exposed to long-term microgravity in space. Changes in blood flow, systemic hormones, and locally produced factors were indicated as important elements contributing to the response of osteoblastic cells to loading, but research in this field still has many questions. Here, the possible biological involvement of thyroid C cells is being investigated. The paper is a comparison between a case of a wild type single mouse and a over-expressing pleiotrophin single mouse exposed to hypogravity conditions during the first animal experiment of long stay in International Space Station (91 days) and three similar mice exposed to hypergravity (2Gs) conditions. We provide evidence that both microgravity and hypergravity induce similar loss of C cells with reduction of calcitonin production. Pleiotrophin over-expression result in some protection against negative effects of gravity change. Potential implication of the gravity mechanic forces in the regulation of bone homeostasis via thyroid equilibrium is discussed.

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Introduction

Parafollicular cells or Thyroid C cells are generally known for producing calcitonin, a hormone involved in calcium homeostasis with hypocalcemic and hypophosphatemic effects but it has been highlighted their role in the production of numerous regulatory peptides such as somatostatin and ghrelin [1], katacalcin I, katacalcin II, gastrin-releasing peptide, thyroliberin and helodermin [2]. Moreover C cells, under regulation by thyrotropin (TSH) because of TSH receptor (TSHR) expression, are involved in the hypothalamic-pituitary-thyroid axis [3]. Accumulating evidence showed that C-cells express thyrotropin releasing hormone (TRH) carrying out paracrine activity on follicular cells and inducing in them TRH-Rs expression [4]. In this way C cells are responsible for intrathyroidal regulation of follicular cells by permitting an interrelationship between the two endocrine populations [5].

Studies on the behavior of the thyroid C cells in follicular pathological conditions are contradictory. Maternal hypothyroidism induced by ^{131}I leaded to the development of hyperplasia and hyperthrophy of calcitonin-positive cells in the pups at the time of birth [6]. Differently, hypothyroidism evoked by propylthiouracil attenuated density of parafollicular cells [7]. In addition less numerous C cells were found in simple and hyperactive goitre in comparison with normal thyroid parenchyma while proliferative changes concerned only follicular cells [8]. It is possible that the variance of results was due to greater complexity of the intrathyroidal regulatory pathway involving several C cell functions.

Space missions are an excellent model to study the simultaneous changes in bone and follicular thyroid metabolism, both affected from C cells. In fact, spaceflight generated a skeletal adaptive response resulting in the loss of bone mass with the change of osteoblast differentiation and morphology [9], calcium metabolism and biochemical markers of bone turnover [10], bone formation and resorption processes [11]. Changes in blood flow, systemic hormones, and locally produced factors were indicated as important elements contributing to the response of osteoblastic cells to loading [9] but research in this field still has many questions. It has been demonstrated that in the longest mice permanence (91 days) on International Space Station (ISS) during the Mice Drawer System (MDS) mission, animals presented a bone loss but transgenic mice over-expressing pleiotrophin (PTN-TG), molecule that produces positive effects on bone turnover, had an osteoblast activity higher than that observed in wild type (WT) mice, indicating that the expression of the PTN during the flight resulted in some protection against microgravity's negative effects [12]. In the same experimental model, the structure of thyroid follicles appeared more organized, TSHR more expressed, cAMP release under TSH stimulation more intense in spaceflight mice than in control animals. The thyroid of PTN-TG mice was characterized by poorly developed follicles that were heterogeneous because of the variable size of both cells and colloidal spaces and the variability increased strongly in space environment

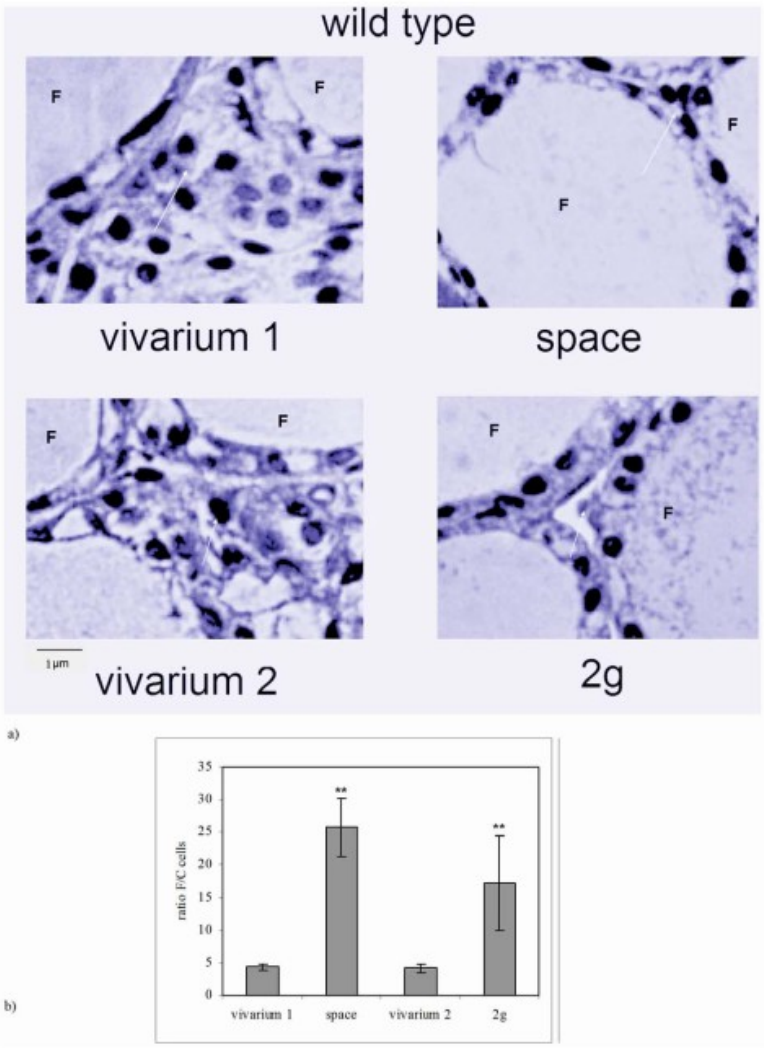

Figure 1. Effect of the gravity change on thyroid tissue of WT animals. a) Morphology analysis of parafollicular thyroid cells. "vivarium 1": mice maintained in vivarium cages (control for experiment in hypogravity); "hypogravity": experimental mouse in space; "vivarium 2": control for experiment in hypergravity; "hypergravity": experimental mice in 2×g centrifuge. Hematoxylin-eosin staining, 40× magnification, 1 µm scale bar. F = follicle. b) Ratio between the number of follicular cells of three follicles delimiting a parafollicular area and the number of cells C in this area. The values are expressed as mean ± SD of three independent fields observed in duplicate (7 and 13 sections). (Significance, **P<0.001 space versus vivarium 1 and 2 g versus vivarium 2).
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together to an increase of TSHR and cAMP although with lower values than those of WT mice [13]. In spite of the existence of data on thyroid follicular cells changes during space missions, no observation has ever been recorded on thyroid parafollicular cells in the space environment. Here we reported the results of the behavior of C cells obtained by using the same mice of the same experimental model of Tavella et al. [12] and Masini et al. [13] to understand their interaction with bone metabolism. To test the role of the physical force of gravity on the modifications obtained during the mission, the experiments were repeated in conditions of hypergravity.

Results

1. How thyroid parafollicular cells sense the change of the gravity

We have previously demonstrated that while in the thyroid gland of WT control mice the follicles had variable size and spatial orientation, spaceflight animals presented a more homogenous

thyroid tissue structure, with ordered follicles and reduction of interfollicular space [14]. Since most species C cells are mainly concentrated in the middle third of each thyroid lobe, the so-called *C-cell region* [15], we have focused the attention on this specific area. Our observations showed that in this area each interfollicular space is delimited by three follicles. Fig. 1a shows the particular of the walls of two adjacent follicles normally structured with numerous interfollicular cells in vivarium 1 (control for the space experiment). It is known that the follicle is surrounded by thyrocytes or follicular cells. The analysis of the cell number in vivarium 1 sample highlighted that the sum of the follicular cells of three follicles delimitating an interfollicular space is 78±9 whereas the number of C cells is 18±3. The ratio between the two cell types is reported in Fig. 1b. In space environment the interfollicular space is strongly reduced (Fig. 1a) and the number of follicular and C cells is 75±6 and 3±2 respectively, by increasing consequently their ratio (Fig. 1b). Thus it is clearly evident that the space environment induces a loss of C cells. To try to discriminate whether this effect was due to the reduction of gravity force or to other factors of the space environment we thought to repeat the experiments in hypergravity condition with the idea of obtaining or opposite results for the principle of opposites or similar results. This would open a whole issue related to the fact that any change of a physical force of gravity would have an impact on cellular function. The results have highlighted that the number of follicular and C cells in the control sample (vivarium 2) is 66±8 and 16±7 respectively, similar to those of vivarium 1 and consequently they have a similar ratio (Fig. 1b). In hypergravity conditions the number of follicular and C cells is 69±9 and 4±3 respectively, by increasing their ratio with respect to vivarium 2 (Fig. 1b). If you compare the results of hypo- and hypergravity it appears evident that they induce a similar effect on the reduction of C cells. Since thyroid C cells are mainly known for producing calcitonin we have performed immunohistochemical analysis with anti-calcitonin antibodies to test C cell function. The results show the immunopositivity in the central regions of the thyroid gland lobes, as expected, of vivarium 1 and vivarium 2 controls (Fig. 2a). Median and range values of surface area are 3,49 (3.86–3,39) mm^2 and 2,77 (3,45–2,71) mm^2 in the vivarium 1 and vivarium 2 respectively. Either in space sample or in 2 g sample the immunopositivity is strongly reduced (Fig. 2a) even if with different values. In fact, in the space environment the immunopositivity is evident in a surface equal to 0,019 (0,015–0,021) mm^2 whereas in 2 g sample the value of surface is 0,39 (0,37–0,43). The ratio between the value of immunopositivity surface and total surface of the thyroid lobe is reported in Fig. 2b. Even if the number of cells C is similar in hypo- and hypergravity, the surface of the positive area to anti-calcitonin antibody is wider in hyperthan in hypogravity. This result allows to suppose that the few cells present are more active in hyper- than in hypogravity.

2. Pleiotrophin keeps thyroid C cells in shape

Since the over expression of the PTN during the spaceflight resulted in some protection against microgravity's negative effects on bone tissue [12], we wanted to investigate whether there was an effect of this protein on thyroid parafollicular cells and the production of calcitonin involved in bone metabolism.

We previously noted that the thyroid of PTN-TG mice were characterized by follicles poorly developed and with variable size of thyrocytes and colloidal spaces [13], suggesting that the overexpression of PTN induces a follicular change. Here we show that the number of C cells is high in both control samples, vivarium 1 and vivarium 2, as occurs in WT mice thyroid, indicating that the PTN over-expression does not influence C cells in the ground (Fig. 3). Differently in both space and 2 g animals, the PTN overexpression reduces strongly the loss of C cells observed in WT mice thyroid. Because of the high irregularity of the thyroid lobe structure is really difficult to make an accurate analysis of the number of follicular and C cells. In fact, in Fig. 4, the vivarium 1 and vivarium 2 samples show follicles altered in shape and size with abnormal light areas with respect to WT samples (Fig. 2), supporting previous results [13]. The labelling for calcitonin is similar to that of WT samples. In the spaceflight animals the size of follicles is greatly heterogeneous with nuclei more evident, supporting previously observation [13] with the positivity for calcitonin lower than that of its control (vivarium 1) but significantly higher than that of WT mice. In this sample the immunopositivity is irregular and spread unevenly and it is very difficult to calculate the surface area occupied. In the 2 g sample you can see the similar results (Fig. 4).

Discussion

In this study, we provide evidence that both microgravity and hypergravity induce similar numeric and functional changes of thyroid parafollicular cells, suggesting a potential implication of the mechanic forces in the regulation of bone homeostasis via thyroid equilibrium. It has been demonstrated that *in vitro* microgravity and hypergravity produce contrary effects. In fact, in Helix lucorum and Pomatias rivulare, statoconia and statoliths grew in number significantly in hypogravity whereas hypergravity caused their massive destruction [16]. Equally, global transcriptional state of Arabidopsis thaliana was influenced in opposite way in the two experimental models [17]. Platelet aggregation and platelet adhesion to von Willebrand factor were significantly decreased after platelets were exposed to simulated microgravity. Conversely, these platelet functions were increased after platelets were exposed to hypergravity [18]. It is possible to think that the effect of gravity changes on a complete (multicellular) organism, where multiple mechanisms of functional regulation are present, is more complex. In addition, in each tissue the balance between mechanical forces, both intra- and extracellular, that determine cell shape and integrity, influences and is influenced by what happens in neighboring cells. Very complex are the mechanisms of metabolic regulations. Bone loss is one of the most important complications for astronauts who are exposed to long-term microgravity in space. It is generally known that microgravity is associated with the loss of bone in astronauts resulting from an exceptional form of disuse together to mineral metabolism alteration and the loss of information that must be communicated to the effector cells that form new bone or destroy old bone mediated by different molecules such as prostaglandin and prostaglandin G/H synthase inducible cyclooxygenase [19]. And so, dependent on the mission length, individual turnover rates, stress, nutrition, fluid shifts, dehydration and bone perfusion, the astronauts undergo osteoporosis characterized by deterioration of bone tissue leading to enhanced bone fragility and to a consequent increase in fracture risk [20]. The development of space osteoporosis is supported by low energy intake, low calcium intake, low plasma 25-hydroxy-vitamin D or low calcitriol levels but dietary calcium and vitamin D do not stabilize bone turnover because markers of bone formation were reduced and markers of bone resorption were increased [21]. So far no data exist, obtained from space missions, regarding the production of calcitonin, a pharmacological inhibitor of osteoclastic bone resorption. And yet, nasal calcitonin treatment provided dual action on osteoporosis and osteoarthritis with significant improvements in quality of life [22].

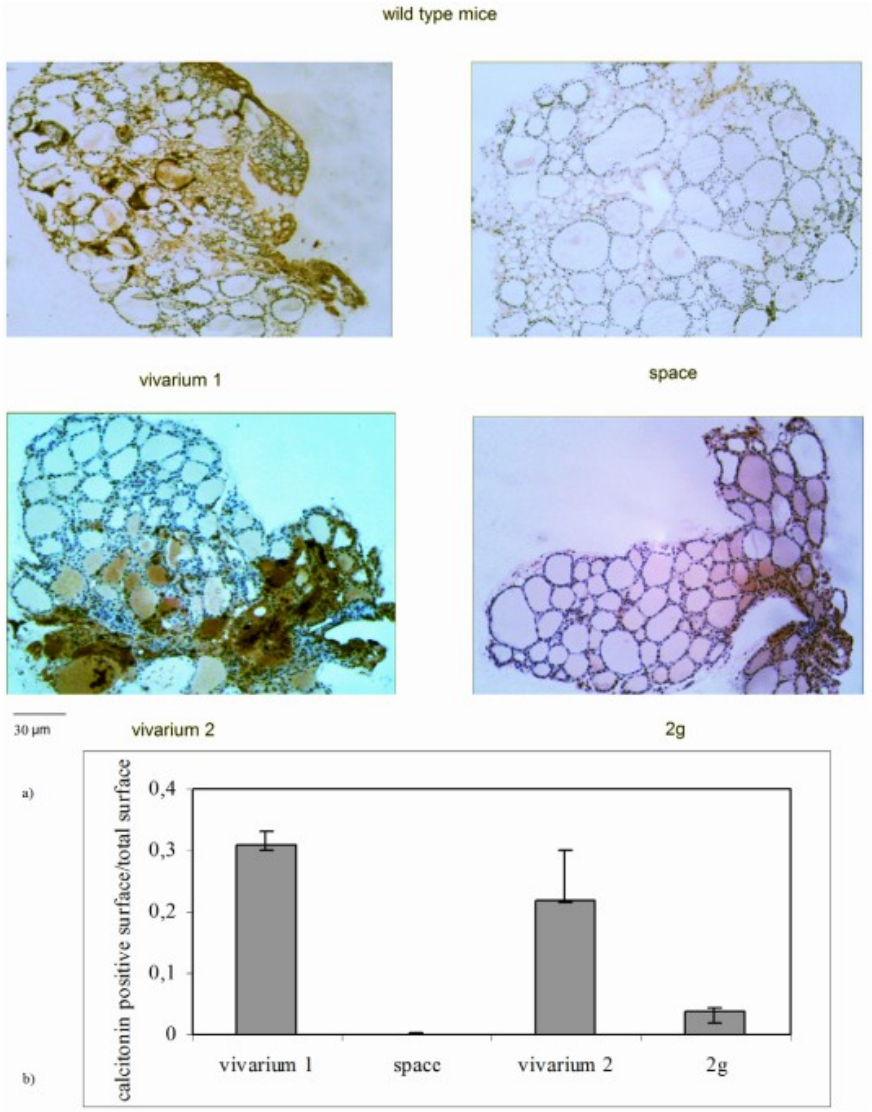

Figure 2. Effect of the gravity change on calcitonin production in WT animals. a) Calcitonin detection in thyroid tissue of WT animals. "vivarium 1": mice maintained in vivarium cages (control for experiment in hypogravity); "hypogravity": experimental mouse in space; "vivarium 2": control for experiment in hypergravity; "hypergravity": experimental mice in 2×g centrifuge. Immunohistochemical staining. 4× magnification, 30 µm scale bar. b) Ratio between the parafollicular surface and total surface of thyroid lobe. The values are expressed as median and range of two sections (8 and 14).
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Here we show the first evidence of the parafollicular cells loss and the reduction calcitonin production in the absence of weight. We propose that the reduction of calcitonin may be involved in osteoporotic damage produced during space missions. Our results clearly indicate that the hypergravity induces a similar effect to that obtained in hypogravity, suggesting that, in any case, the change of mechanical force results in a stress condition with the same damage on thyroid parafollicular cells. It has been demonstrated that short term exposure to hypergravity induces significant reductions in the thicknesses of cortical bone at the anterior (13%) and medial regions (15%) of the mid-diaphysis but femoral bone density, collagen and calcium concentrations are unaltered [23]. In addition the content of mature, stable bone collagen cross-links hydroxylysylpyridinoline (HP), lysylpyridinoline (LP), are significantly greater in bones from centrifuged animals than in bones from control animals [23]. Since it was not

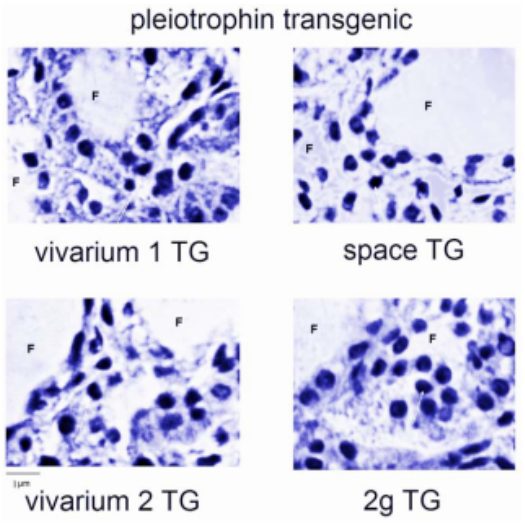

Figure 3. Effect of the gravity change on thyroid tissue of PTN-TG animals. Morphology analysis of parafollicular thyroid cells. "vivarium 1": mice maintained in vivarium cages (control for experiment in hypogravity); "hypogravity": experimental mouse in space; "vivarium 2": control for experiment in hypergravity; "hypergravity": experimental mice in 2×g centrifuge. Hematoxylin-eosin staining, 40× magnification, 1 μm scale bar, F = follicle.
doi:10.1371/journal.pone.0048518.g003

possible to measure the bone turnover markers in our study because of the unavailability of the blood of animals, we do not know at the time the effects on bone metabolism of long stay in hypergravity conditions.

Since the spatial integration of follicular and parafollicular cells and functional coordination of both epithelial cell lines exists in normal conditions [24], it is possible that modifications of follicular cells during space mission [13] and in hyper-gravity conditions, regulated in turn by hypothalamus, are responsible for parafollicular cell changes. The loss of calcitonin in hypergravity rather than act on bone metabolism may play a role in the intrathyroidal regulatory pathway of thyroid hormone synthesis. Here we report that over expression of PTN, or osteoblast-stimulating factor 1 or heparin-binding growth-associated molecule [25], limits the damage produced by hypo- or hypergravity conditions. Tavella et al. [12], discussed that during flight WT mice tend to lose more bone trabeculae than PTN-TG mice, suggesting that the over expression of the PTN exerts some protection on the skeleton against the bone loss consequent to the microgravity exposure but how PTN transgene could prevent in the transgenic mice bone tissue cell morphology alteration observed in WT bones is not defined. The authors shown that the reduction in the expression of collagen type I and osteocalcin in PTN-TG was less than in the samples from WT mice. We propose a reduction bone resorption due to the higher level calcitonin expression in PTN-TG mice in comparison with WT mice that could participate to the protective effect of PTN overexpression on the bone damage. To confirm our results it would be really important to know the blood levels of calcitonin in the hypogravity and hypergravity of WT or PTN-Tg mice but in this study we have participated in a "Tissue Sharing Program" in which every group has collected and studied the organ of his interest. We have taken the thyroids which were the subject of our study, while blood was collected from other groups for different analyses. Future space missions and hypergravity experiments could clarify this aspect of the study.

Materials and Methods

Experimental design and animal care

All experimental procedures were authorized by the Public Veterinary Health Department of the Italian Ministry of Health. The experiment was also conducted in accordance with the regulations for the care and use of laboratory animals and with the guidelines of the Japanese Physiological Society. Furthermore, this study was also approved by the Committee on Animal Care and Use at Graduate School of Medicine, Osaka University (No. 22-071). Finally, the protocol utilized in the study has been authorized by the Public Veterinary Health Department of the Italian Ministry of Health. All experiments were carried out using male C57BL/10J mice (8 weeks old).

Hypogravity experiment

WT and PTN-TG mice (n = 3 each) were individually housed in the Mouse Drawer System (MDS), a 11.6×9.8×8.4 cm payload developed by Thales-Alenia Space Italy and all treatments were performed as previously reported [13]. Food and water were supplied *ad libitum*. The MDS, loaded with 3 WT and 3 TG mice, was launched in the Space Shuttle Discovery, within the Space Transport System (STS)-128 mission, on August 28, 2009. It was then housed in Japanese Experimental Module (Kibou) on the ISS until its return to the Earth by Space Shuttle Atlantis (STS-129 mission) on November 27, 2009. Only 1 WT and 2 TG mice returned to the Earth alive after 91 days of space flight.

Thyroids were sampled bilaterally from each mouse killed by inhalation of carbon dioxide at the Life Sciences Support Facility of Kennedy Space Center within 3–4 hours after landing and either processed or frozen immediately, according to the various experimental protocols. The procedure was approved by the IACUC protocol n° FLT-09-070(KSC).

After the spaceflight experiment, the on-ground experiment was also carried out at the Vivarium of the Advanced Biotechnology Center in Genova, Italy. One group of mice with the same species, sex, and age were housed in normal vivarium cage as the laboratory control (Vivarium1). Amount of food and water supplementation and environmental conditions were simulated as the flight group. After 3 months, thyroids were sampled bilaterally from 3 WT and 3 TG mice and treated as above reported for spaceflight mice.

Hypergravity experiment

WT and PTN-TG mice (n = 3 each) of the same strain as those used in hypogravity experiments, were maintained in hypergravity, with conditions similar to the MDS experiment, in a 2×g centrifuge in the laboratory of Dr. Y. Ohira at the Osaka University, Osaka, Japan. Control mice were similar to those reported in hypogravity experiment (Vivarium 2). Animals were treated, and thyroids were obtained and processed with the same procedures used in the hypogravity/space experiments.

Thyroid tissue treatment

The thyroid lobes were fixed in 4% neutral phosphate-buffered formaldehyde solution for 24 h as previously reported [13]. Thyroids were dropped with essentially random orientation in paraffin. The paraffin blocks were sectioned into 4-μm-thick sections. All sections were mounted on silan-coated glass slides. Each slide contained a pair of sections at a distance equal to

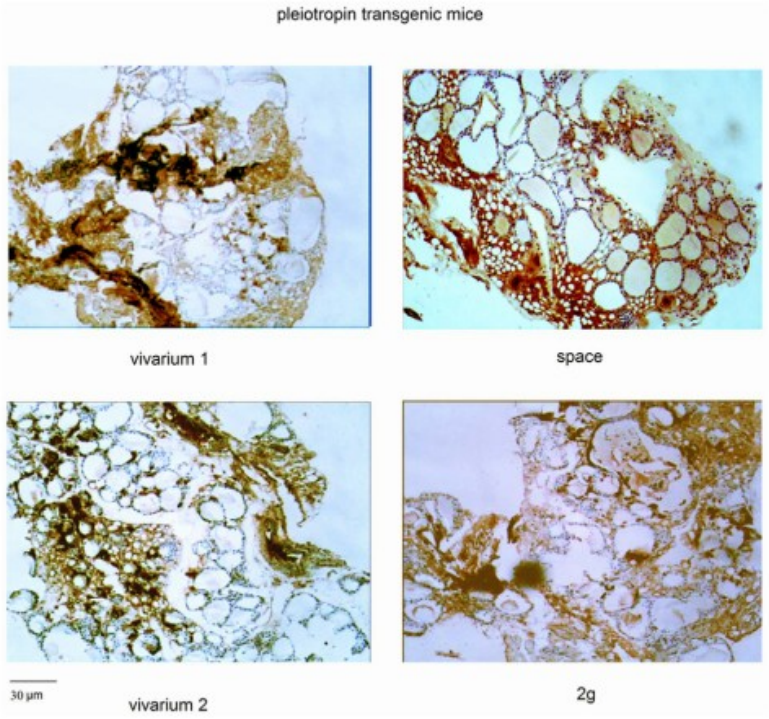

Figure 4. Effect of the gravity change on calcitonin production in WT animals. Calcitonin detection in thyroid tissue. "vivarium 1": mice maintained in vivarium cages (control for experiment in hypogravity); "hypogravity": experimental mouse in space; "vivarium 2": control for experiment in hypergravity; "hypergravity": experimental mice in 2×g centrifuge. Immunohistochemical staining. 4× magnification, 30 µm scale bar.
doi:10.1371/journal.pone.0048518.g004

140 µm. Between 7 and 14 pairs of sections were sampled excluding the first and the last; 7 and 13 sections were used for morphological analysis whereas 8 and 14 sections were used for immunohistochemical analysis. Tissue sections were deparaffinized and rehydrated through a series of xylene and ethanol washes.

Morphological analysis

The sections were stained by the hematoxylin-eosin (Chroma-Gesellschaft, Germany) staining method and investigated for parafollicular cells detection by using inverted microscopy EUROMEX FE 2935 (ED Amhem, The Netherland) equipped with a CMEX 5000 camera system (40× magnification).

Immunohistochemical analysis

For immunohistochemical analysis Bond Dewax solution was used for removal of paraffin from tissue sections before rehydration and immunostaining on the Bond automated system (Leica Biosystems Newcastle Ltd, UK) as previously reported [14]. Immunostaining for calcitonin detection was performed according to Bancroft and Stevens [15] by using NCL-L-calcitonin and Bond Polymer Refine Detection - Leica Biosystems ((Newcastle Ltd, UK). The observations were performed by using inverted microscopy EUROMEX FE 2935 (ED Amhem, The Netherland) equipped with a CMEX 5000 camera system (4× magnification). The analysis of the tissue section size was performed by ImageFocus software.

Statistical analysis

The experiments have been conducted on the thyroid of: 1 animal for the hypogravity experiment (the only returned alive from the mission), 3 control animals for the hypogravity experiment (vivarium 1); 3 animals for the hypergravity experiment; 3 control animals for the hypergravity experiment (vivarium 2). For morphological analysis, the means ± SD of 3 fields of the 7 and 13 sections were given. The significance of the differences between the data was checked by Student's t-test. For immunohistochemical analysis the medians and ranges of 8 and 14 sections were given.

Author Contributions

Conceived and designed the experiments: EA FC FSAI. Performed the experiments: RS AL RL EL IF SC. Analyzed the data: EA IF FC. Contributed reagents/materials/analysis tools: FC FSAI. Wrote the paper: EA FSAI.

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COMMENTO DEL VI° LAVORO

I nostri risultati hanno mostrato che in ipogravità vi è una forte riduzione dello spazio interfollicolare sia in ipo e sia in ipergravità con una riduzione del numero delle cellule C.

Con l'immunoistochimica abbiamo riscontrato una forte riduzione dell'immunopositività per la calcitonina, più intensa in ipo che in ipergravità.

5- INTRODUZIONE AL V° LAVORO

Al fine di valutare le possibili modificazioni tiroidee che potessero indurre un danno negli astronauti abbiamo valutato il rapporto tra la funzionalità delle cellule follicolari e quelle parafollicolari in topi WT e TG mantenuti per 91 giorni in MDS in ISS, come sopra riportato.

Structural modifications in Mammalian Thyroids exposed to Hypogravity – Importance for Astronauts

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In mammals, the persistence of health condition requires the maintenance of a homeostasis which largely depends on the correct coordination of the endocrine system. This equilibrium is very likely to be subject to significant perturbations in space, as already proven by the several pathological alterations reported in astronauts. Among endocrine glands, thyroid has proven to have an important role in the metabolism of cardiovascular, musculoskeletal, immune and nervous system. In our previous experiences in space research we have performed several *in vitro* studies on epithelial thyroid cells that stimulating us to perform further in vivo studies. Thus we have analysed the behaviour of thyroid in space-exposed mice by joining the "Tissue Sharing" experiment coordinated by R. Cancedda, thus participating to the Space Shuttle/ISS 90 days mission, in which animals were housed inside the Mice Drawer System (MDS) facility developed by the Italian Space Agency. C57BL/10J mice, 8 weeks old at launch, either wild type (WT) or osteoblast stimulating factor (OST) transgenic (TG), were used as experimental models. Mice of the same species, sex, and age were housed in MDS for 3 months as ground controls (Fig.1). Spaceflight animals showed a more homogenous structure of thyroid tissue, as compared to the control samples, with a prevalence of ordered and large follicles in which follicular thyrocytes were thicker and the nuclear volumes appeared increased with consequent change of thyroid epithelium vs. colloid volumetric ratio (Fig. 1a). In addition, thyroids were characterized by a loss of parafollicular cells with reduction of calcitonin production (Fig.1b).

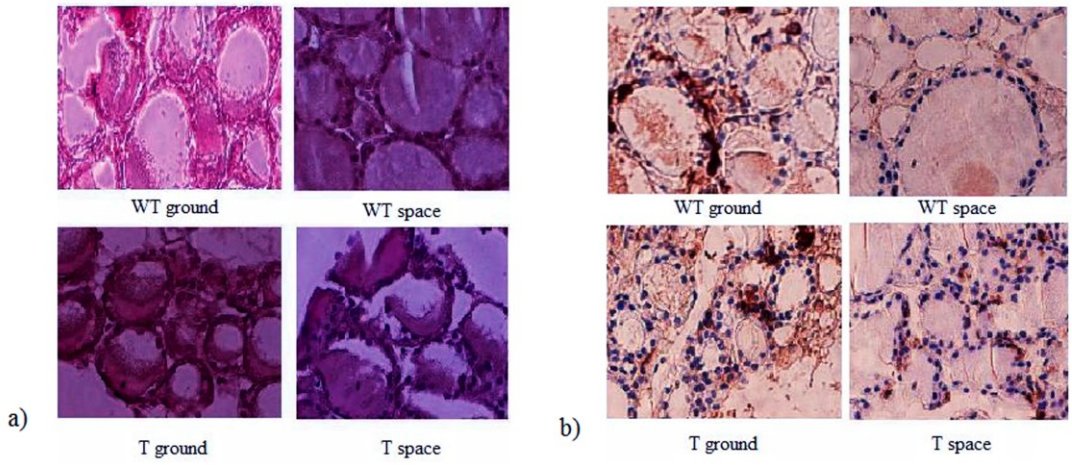

Fig.1 Change of thyroid in space environment. a) Morphology analysis of thyroid tissues of ground and space WT and TG mice, hematoxylin-eosin staining, 4x magnification; b) Immunohistochemical staining for calcitonin detection. 4x magnification

In the space environment, thyroids expressed more thyrotropin-receptor (TSHR), sphingomyelinase (SMase) and sphingomyelin-synthase (SM-synthase) (Fig.2)

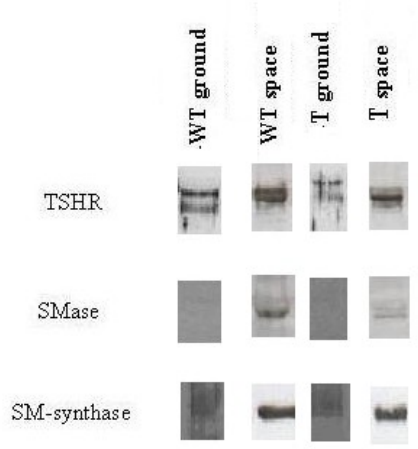

Fig.2 Thyrotropin receptor (TSHR), sphingomyelinase (SMase) and sphingomyelin-synthase (SM-synthase) in thyroid tissues of ground and space WT and TG mice. The study was performed by immunoblotting analysis by using specific antibodies and the bands were identified in relation to the position of molecular size standards.

Interestingly, SMase moved from the nucleus to the cytoplasm and cell membrane where interacted with TSHR (Fig.3)

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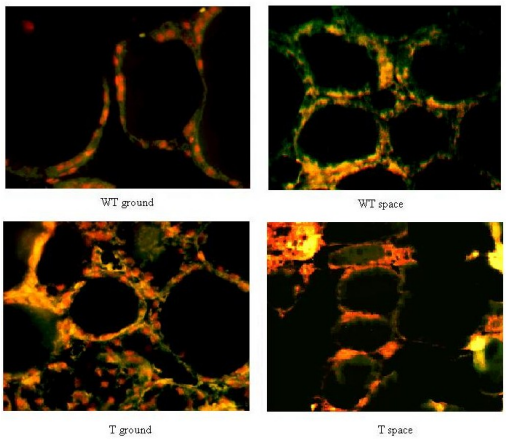

Fig.3 Fluorescence immunostaining of TSHR (green) and SMase (red) by using specific antibodies in control and space WT and TG mice.

There, significant fraction of TSHRs was constitutively associated with lipid microdomains rich in sphingomyelin (SM) and cholesterol (CHO) in which TSH-TSHR interaction occurred. The migration of SMase in cell membrane degraded SM by freeing ceramide and constituting a critical moment in the initiation of signal transduction. Thus, the space environment appears to induce in vivo an hyperfunctionality of follicular cells at the expense of the parafollicular ones. On the basis of all findings obtained in our study, we suggest that long-term exposure to real microgravity environment induces structural/functional changes of the thyroid gland which significantly affect the endocrine homeostasis of astronauts.

COMMENTO DEL V° LAVORO

I risultati hanno mostrato che l'ambiente spaziale induce *in vivo* un' iperfunzionalità delle cellule follicolari a spese di quelle parafollicolari. Infatti in ipogravità i follicoli appaiono più larghi ed ordinati e circondati da tireociti più alti con un conseguente cambiamento del rapporto volumetrico epiteliotiroideo-colloide.

In associazione all'over-espressione della SMasi e SMsintasi, come riportato anche nel lavoro precedente, si ha un over espressione del TSHr, di notevole rilievo considerato che tale recettore si localizza in microdomini ricchi di SM e CHO nonché di SMasi e SMsintasi.

Con l'immunoflorescenza è apparso chiaro che la SMasi muovendosi dal nucleo al citoplasma, come riportato anche nel lavoro precedente, interagisce con il TSHr.

Sulla base di tali osservazioni, abbiamo suggerito, che l'esposizione a lungo termine in un ambiente di reale ipogravità induce cambiamenti strutturali e funzionali della ghiandola tiroidea che procurano significativi effetti sull'omeostasi endocrina degli astronauti.

6- INTRODUZIONE AL VI° LAVORO

Per valutare se le modificazioni della ghiandola titoidea osservate al rientro dalla missione spaziale fossero in parte dovute all'ambiente confinato nel quale gli animali sono stati costretti a vivere, per riprodurre l'ambiente confinato degli astronauti abbiamo valutato le modificazioni strutturali e funzionali della ghiandola in topi mantenuti in MDS a terra.

The health of the astronauts in the long-duration space flight: the importance of the thyroid gland

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Thyroid hormones play a key role in energy homeostasis of humans. Their production and secretion by the thyroid gland is regulated via the hypothalamus-pituitary-thyroid axis. The hormones are transported into the cell of various organs where they can bind to the thyroid hormone receptors. In this way thyroid gland control cardiovascular, musculoskeletal, nervous and immune systems activity. During space flights, astronauts live in conditions different from those in which they normally are on the ground, that is, living in a confined environment and in a space environment. The aim of our study was to verify the effect of both these conditions on the thyroid gland of mice. At this end we participated to the Mouse Drawer System (MDS) Tissue Sharing Program and we performed experiments in mice maintained onboard the International Space Station during the long-duration (91 days) exploration mission STS-129. In MDS animals were individually housed in cages equipped by food bars, drinking valves for water delivery, cameras for video observation, white and infrared LED's for illumination and sensors for air composition monitoring and control. Separated cages permit olfactory but not physical contact between animals. Mice in MDS on ground were used to study the effect of confinement whereas mice maintained in the Vivarium of the Advanced Biotechnology Center in Genova, Italy, were used as controls. The structure and function of thyroid gland were analysed by microscopy, immunoblotting, immunofluorescence and immunoistochemistry techniques. Results have shown that confinement changes follicular and parafollicular cells which produce T3-T4 and calcitonin hormones, respectively. Distribution of thyrotropin receptor, caveolin1, sphingomyelinase and sphingomyelin-synthase is different in comparison to those of control animals. Differences are much more marked in animals which had participated in the space mission. Our results clearly indicate that the observed changes of the thyroid gland after the re-entry from space mission are the sum of the effects of the confinement and the space environment.

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Research Article

Reinterpretation of Mouse Thyroid Changes under Space Conditions: The Contribution of Confinement to Damage

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Abstract

During space missions, astronauts work in a state of separation from their daily social environment and in physical confinement. It has been shown that confinement influences mood and brain cortical activity, but no data has been obtained with regard to its effect on the thyroid gland, the structure and function of which change during spaceflights. Here, we report the results of a study on the effects of confinement on mouse thyroid, which was implemented with the Mice Drawer System Facility maintained on the ground, a system used for spaceflight experiments. The results show that confinement changes the microscopic structure of the thyroid gland and that it exhibits symptoms similar to those that result from physiological and/or pathological hyperfunction. What is left unchanged, however, is the sphingomyelinase-thyrotropin receptor relationship, which is important for thyrotropin response with a consequential production of hormones that act on the metabolism of almost all tissues and reduces the production of calcitonin, a hormone involved in bone metabolism. During space missions, the overexpression of pleiotrophin, a widespread cytokine up-regulated after tissue injury that acts on bone remodeling, attenuates changes to the thyroid that are spaceflight-dependent; therefore we studied the thyroids of pleiotrophin-transgenic mice in the Mice Drawer System Facility. In confinement, pleiotrophin overexpression does not protect from the loss of calcitonin. The contribution of confinement to thyroid damage during spaceflights is discussed. Key Words: Calcitonin—Confinement—Sphingomyelinase—Thyroid—Thyrotropin receptor. Astrobiology 14, xxx–xxx.

1. Introduction

During space missions, astronauts are separated from their daily life and forced to live in a confined space for long periods of time (Palinkas, 2001). It is generally believed that confinement represents a potential hazardous condition for acute and/or traumatic physical injury (Yue *et al.,* 2012). It induces the reduction of hearing and balance (Dechesne *et al.,* 1993) and changes the mood and brain cortical activity (Schneider *et al.,* 2010), mental performance (Manzey and Lorenz, 1998), and brain electrical activity (Lorenz *et al.,* 1996). Little is known about the effects of confinement on the body's equilibrium over long periods of time, which, under such conditions, is very likely to be subject to significant perturbations in space, as has already been reported with regard to pathological variations in the bodily functions of astronauts (Rowe, 2009; Smith *et al.,* 2012; Mermel, 2013). The body's equilibrium largely depends on proper coordination of the endocrine system (Michelson *et al.,* 1994). Since the thyroid is an endocrine gland that produces hormones that control the metabolism of almost all tissues, thyroid function impairment may be explained by tissue damage astronauts suffer during space missions (Masini *et al.,* 2012). The thyroid gland is composed of spherical follicles surrounded by a single layer of thyroid epithelial cells (thyrocytes) that secrete T_3 and T_4 hormones that control metabolism of the cardiovascular, musculoskeletal, immune, and nervous systems. Also, parafollicular cells (C cells) in the thyroid secrete calcitonin, which acts on bone metabolism. In the present study, we investigated the role of confinement on changes in the thyroid gland. Previously, we studied the effect of the space environment on the thyroid of mice that were maintained for 91 days on board the International Space Station (ISS). This was in participation with

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the Tissue Sharing team, which was led by R. Cancedda (Cancedda *et al.,* 2012). We demonstrated that, in comparison with control animals, thyroids of spaceflight animals had a more homogenous structure; produced more cAMP; and expressed more thyrotropin receptor (TSHR) and caveolin1 (Masini *et al.,* 2012), sphingomyelinase (SMase), and sphingomyelin-synthase (Albi *et al.,* 2012a). Spaceflight animal thyroids, however, were also characterized by a loss of C cells with a reduction in calcitonin production (Albi *et al.,* 2012b). In these experiments, the animals were maintained during the flight in the Mice Drawer System (MDS), a facility built by Thales Alenia Space-Italy for the Agenzia Spaziale Italiana. As reported by R. Cancedda *et al.* (2012), the mice were hosted in the Mice Chamber, which was divided into two habitats. Each habitat permitted accommodation of the items necessary to provide three individually housed mice with basic services, such as three metallic cages, three food envelopes, three drinking valves for water delivery, three cameras for video observation, white and infrared LEDs for illumination, and sensors for air composition monitoring and control. The cages had grids in all four walls, which permitted olfactory, but not physical, contact between animals. In our previous studies (Albi *et al.,* 2012a, 2012b; Masini *et al.,* 2012), we did not consider the possibility that the results obtained after the return from space might have been due, in part, to the effect of confinement. Here, we focused our attention on the difference between the thyroids of mice that were housed for 3 months in the MDS and those of mice housed for the same period of time in normal vivarium cages at the Vivarium of the Advanced Biotechnology Center in Genoa, Italy.

2. Materials and Methods

Experimental design, authorization of experimental procedures, and animal care were as previously reported (Albi *et al.,* 2012b; Masini *et al.,* 2012).

After isolation of the thyroid gland, the right lobes were used for the morphology analysis with and without thyroid-stimulating hormone (TSH) treatment (Masini *et al.,* 2012), whereas the left lobes were used for TSHR-SMase (Albi *et al.,* 2012a) and calcitonin (Albi *et al.,* 2012b) detection. Sections were prepared, stained, investigated, and analyzed as previously reported (Albi *et al.,* 2012a, 2012b; Masini *et al.,* 2012).

3. Results

3.1. Confinement influences the response of thyrocytes to thyroid-stimulating hormone treatment

Microscopy analysis, performed on histological microsections subjected to hematoxylin-eosin staining, showed that the size of follicles did not change with confinement but that thyrocytes surrounding the colloid lost their disposal in a continuous rim and responded to TSH treatment in a different way compared to what would occur in animals maintained in the laboratory (Fig. 1). In fact, in the thyroids of control mice the height of the thyrocytes was $1.52 \pm 0.06\ \mu$m, and this remained constant after 10^{-8} *M* TSH treatment and increased 2.13 times with 10^{-7} *M* TSH ($P < 0.01$ versus control). In the MDS mice, the height of the thyrocytes was $1.28 \pm 0.14\ \mu$m, and this increased 2.00 and 3.18 times with 10^{-8} *M* TSH ($P < 0.01$ versus control) and

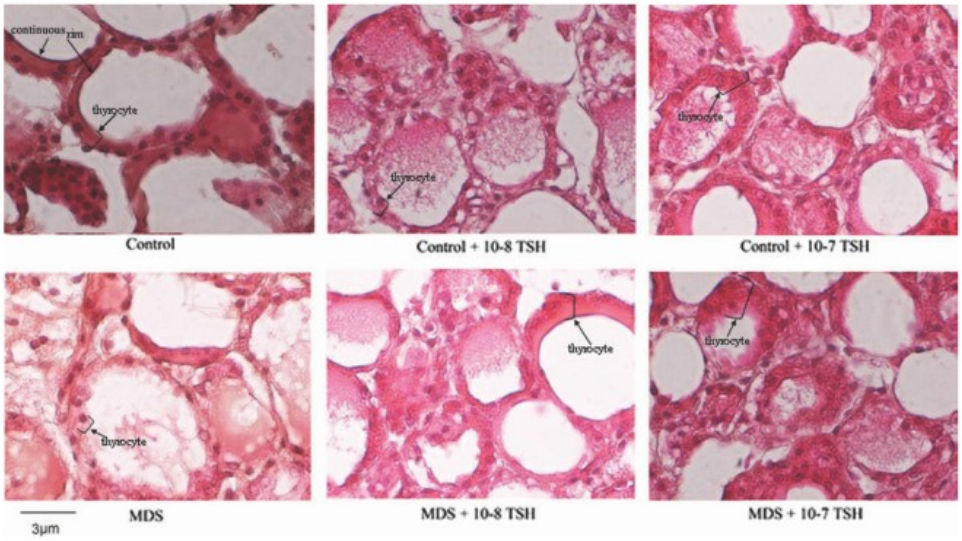

FIG. 1. Confinement influences the response of thyroid tissue to TSH treatment. Morphology analysis of thyroid tissue of mice maintained in vivarium cages (control) and hosted in the MDS. The samples were treated with 10^{-8} and 10^{-7} *M* TSH. Five pairs of sections were sampled, excluding the first and the last; sections 2, 3, and 4 were used. Hematoxylin-eosin staining, 40× magnification, 3 μm scale bar. (Color images available online at www.liebertlonline.com/ast)

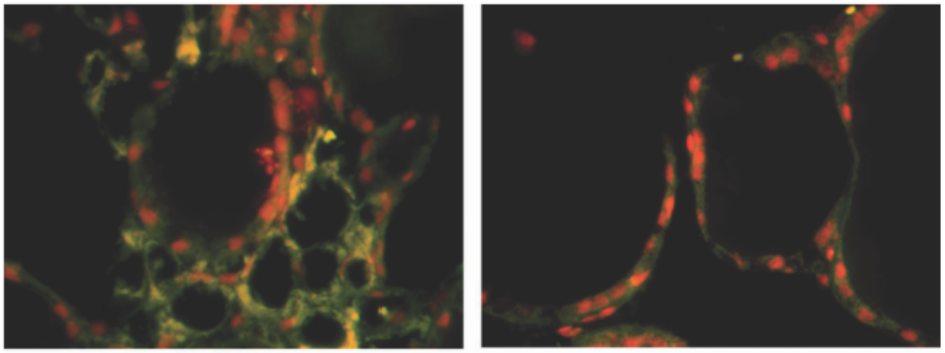

FIG. 2. Effect of confinement on TSHR and SMase. Immunostaining of TSHR and SMase by using specific antibodies in thyroid tissues of mice maintained in vivarium cages (control) and in the MDS. For fluorescence were used fluorochrome-conjugated secondary antibodies, tetramethylrhodamine isothiocyanate (TRITC) for SMase (red) and fluorescein isothiocyanate (FITC) for TSHR (green). (Color images available online at www.liebertlonline.com/ast)

10^{-7} *M* TSH ($P<0.01$ versus control), respectively. Thus, after TSH treatment the thyrocyte/colloid volumetric ratio increased more in the MDS mice than in the controls. This effect was particularly evident in thyroid tissue treated with 10^{-7} *M* TSH (Fig. 1). Since the response to TSH is dependent on TSHR (Masini *et al.,* 2012) and on signal transduction via SMase (Albi *et al.,* 2012a), we then studied the contribution of confinement to the modification of TSHR-SMase. We used the merge image to highlight the relationship between the two proteins (Fig. 2). In MDS mice, the intensity of staining of both TSHR and SMase was similar to that of the control and did not show therefore an increase of expression of the two proteins. In addition, in both control and MDS mice SMase was localized in the nucleus and TSHR in the cell membrane (Fig. 2).

3.2. Pleiotrophin does not protect the thyroid gland from confinement-induced calcitonin reduction

Immunohistochemical analysis showed that, in wild-type (WT) mice housed in the MDS, the parafollicular spaces were more irregular than those of control mice (Fig. 3). The number of C cells was 15 ± 4 in control mice and 4 ± 3 in MDS mice ($P<0.01$ versus control). Immunohistochemical analysis of calcitonin showed that in control mice the immunopositivity covered a $3.21\pm0.77\,mm^2$ surface area, whereas in MDS mice its value was $1.3\pm0.52\,mm^2$ ($P<0.01$ versus control). The overexpression of pleiotrophin (PTN) did not protect the thyroid from the loss of C cell number and the labeling for calcitonin (Fig. 2).

4. Discussion

It is known that, in the blood of astronauts in space, the level of TSH is increased (Leach *et al.,* 1997) and that of thyroid hormones is lowered (Strollo, 1999). The study of the possible alteration of the thyroid gland that may be responsible for the hormonal changes would require the removal of the gland in humans. Thus, we had the occasion to participate in the Tissue Sharing program led by R. Cancedda (Cancedda *et al.,* 2012), and we had access to the thyroids of mice that had been exposed to the space environment for 91 days on board the ISS and inside the MDS as reported above (Masini *et al.,* 2012). We have shown that the structure of the thyroid gland changes in the space environment; the follicles become more rounded and are surrounded by higher thyrocytes with larger nuclei such that the volumetric ratio thyrocyte/colloid becomes higher, which is the index of physiological and/or pathological hyperfunction, as supported by the highest production of cAMP upon TSH stimulation (Masini *et al.,* 2012). The production of cAMP in MDS mice is similar to that of mice kept in the laboratory (Masini *et al.,* 2012). Therefore, apparently the confinement in the MDS does not have an influence on the TSH response. However, the possibility that the confinement of mice in the MDS system might induce structural and/or molecular changes could not be excluded. Here, we demonstrated that the confinement-induced structural changes of thyrocytes upon TSH treatment could be the basis for the subsequent functional changes obtained in microgravity with cAMP increase. In addition, we have previously demonstrated with regard to mice that the long-term exposure to real microgravity induces an increase of expression of TSHR (Masini *et al.,* 2012) and SMase and the transportation nucleus-cytoplasm of SMase (Albi *et al.,* 2012a) in comparison with that of control mice. Here, we studied the contribution of confinement on the modification of TSHR-SMase. Our data demonstrate clearly that the changes of TSHR and SMase present upon the return to Earth of a space mission (Albi *et al.,* 2012a; Masini *et al.,* 2012) were due exclusively to the space environment. In space, SMase moved from the nucleus, where it acts on duplication and transcription processes (Albi *et al.,* 2003; Rossi *et al.,* 2007), to the cytoplasm and the cell membrane, where it probably freed ceramide from sphingomyelin that was present in lipid microdomains that contained TSHR and, after TSH-TSHR interaction, induced transduction of the signal in the cells (Albi *et al.,* 2011).

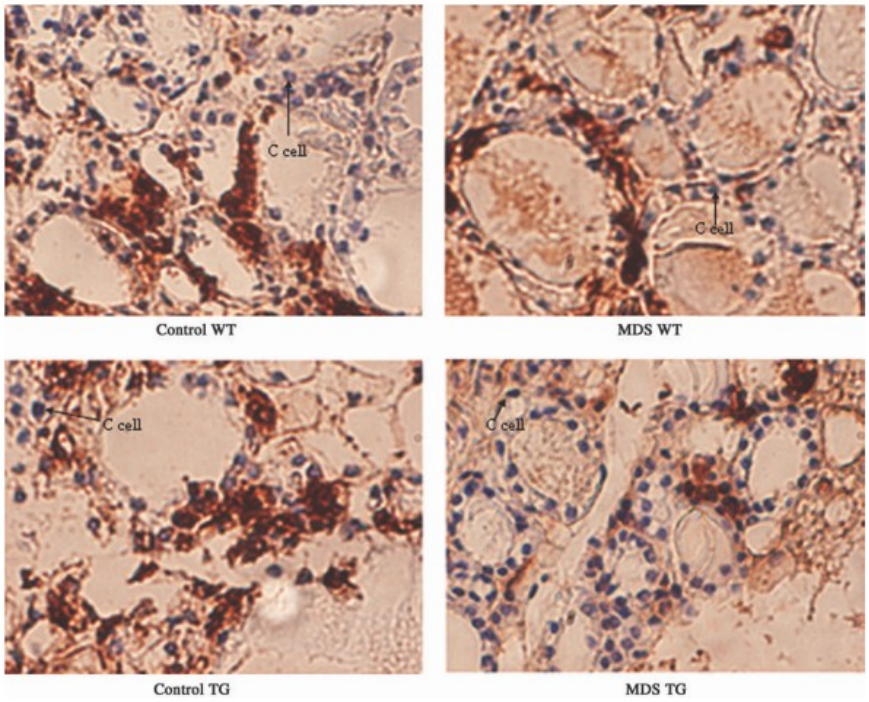

FIG. 3. Effect of confinement on calcitonin production in WT and PTN-TG animals. Calcitonin detection in thyroid tissue of WT and PTN-TG mice maintained in vivarium cages (control) and in the MDS. Immunostaining for calcitonin detection was performed by using NCL-L-calcitonin and Bond Polymer Refine Detection—Leica Biosystems (Newcastle Ltd., UK). In brown, calcitonin immunohistochemical staining. 40× magnification, 3 μm scale bar. (Color images available online at www.liebertlonline.com/ast)

In addition, during spaceflight, while the follicles changed structure, the interfollicular spaces lost C cells resulting in reduced production of calcitonin, which is important for bone metabolism (Albi *et al.,* 2012b). Thus, in the present study, we examined the contribution of confinement to this damage. Since PTN acts on bone remodeling (Imai *et al.,* 2009), we studied calcitonin in WT and pleiotrophin-transgenic (PTN-TG) mice. Our results indicate that, after mice experienced a long stay in the ISS, the reduction of calcitonin observed in WT and PTN-TG mice (Albi *et al.,* 2012b) was observed to be partially present in mice maintained in the MDS on the ground.

5. Conclusion

In conclusion, we have shown for the first time that some of the thyroid changes in mice during spaceflight, which we have previously ascribed to the space environment, are actually the result of confinement. Until now, little has been known about the influence of a confined environment on the hypothalamic-pituitary-thyroid axis or on changes in coordination of the endocrine system. Our results highlight that confinement acts specifically on the structure of the thyroid, stimulates thyrocytes, and inhibits C cells. Our results here may apply to the study of humans in that the structure and function of the human thyroid are very similar to those of the mouse thyroid. Mice in the MDS are isolated physically such that freedom of movement is reduced in much the same way as the freedom of astronauts is reduced during space missions (Cancedda *et al.,* 2012). It would, therefore, be of great benefit to study effective countermeasures to help maintain good health and performance in astronauts who are exposed to a confined environment over long periods of time.

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Glossary

Mice Drawer System (MDS), a facility built by Thales Alenia Space-Italy for the Agenzia Spaziale Italiana.

Pleiotrophin (PTN), also called HARP, HB-GAM, or OSF-1, is a molecule expressed by several cell types during early differentiation and up-regulated after tissue injury, involved in bone remodeling.

Pleiotrophin over-expressing mice, animals in whose genome pleiotrophin has been inserted as a foreign gene.

Sphingomyelinase (SMase), an enzyme that degrades sphingomyelin. It is localized in cell nuclei and in lipid microdomains of cell membranes composed by sphingomyelin and cholesterol that act as platform for TSHR.

Transgenic (TG) mice, animals with insertion of foreign DNA or removal of native DNA. The technique permits the manipulation of genetic material in the whole animal to study physiological and pathological processes.

TSHR, receptor for thyroid-stimulating hormone (TSH) released by the anterior pituitary gland, located at the base of the brain, that regulates the activity of the thyroid gland.

Wild-type (WT) mice, animals with gene composition commonly present in nature.

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COMMENTO DEL VI° LAVORO

I risultati hanno chiaramente indicato che i cambiamenti osservati della ghiandola tiroidea al rientro della missione spaziale sono la somma degli effetti del confinamento e dell'ambiente spaziale.

7- INTRODUZIONE AL VII° LAVORO

È stato dimostrato che il TSHr, è un recettore associato alla proteina G e interagisce con la tirotropina nei microdomini di membrana ricchi di SM e CHO. I nostri studi effettuati in condizioni di ipogravità avevano dimostrato che l'interazione TSH-TSHr-microdomini di membrana era fortemente perturbata in condizioni di ipogravità.

Nel presente lavoro abbiamo studiato se i complessi effetti meccanici di forze opposte all'ipogravità, come quelle dell'ipergravità, potessero indurre modificazioni di TSHr CHO e SM nella ghiandola tiroidea *in vivo.*

A tale scopo topi della stessa razza e della stessa età di quelli utilizzati in ipogravità sono stati posti in una centrifuga a 2g, come riportato in materiale e metodi.

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Hypergravity delocalizes thyrotropin receptor (650.3)

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Abstract

Thyrotropin receptor (TSHR) is a a heterodimer G protein-coupled receptor constituted by an α extracellular subunit (53 kDa) interacting with thyrotropin (TSH) and a broad β transmembrane and intracellular subunit (30-42 kDa) held together by disulphide bridges. TSHR are localized in the basolateral membrane rich in lipid rafts, microdomains with high level of cholesterol and

sphingolipids, that provide a platform for the assembly of signaling complexes after TSH-TSHR interaction. This interaction was perturbed in hypogravity during parabolic flights and longer missions in Space on board the ISS. In the present work the influence of complex mechanical effects of hypergravity on TSHR, cholesterol and sphingolipid in thyroid gland in vivo was investigated. Seven mice were maintained in hypergravity in a 2g centrifuge. Our results show that hypergravity up-regulated 65% TSHR. TSH treatment did not cause significant changes in the thyroid gland of the control mice and induced about 50% reduction of the α subunit TSHR of 2g animal. TSH-stimulated cAMP production was similar in control and 2g samples, indicating that even if TSHR is up-regulated in hypergravity, the loss of its α subunit that binds TSH was responsible for the lack of the expected increased response to hormonal stimulation. Immunofluorescence analysis of TSHR demonstrated that in control samples the receptor was present on the surface of thyrocytes that surrounded follicles with a precise location, whereas in 2g samples the fluorescent signal was higher and spread over the entire surface of thyrocytes. The UFLC-MS/MS study on lipid fraction of thyroid tissue showed that sphingomyelin and ceramide species remained unchanged whereas cholesterol was reduced 49% in 2g in comparison with control samples. We thus suggest that gravity, by inducing a significant loss of cholesterol, perturbed lipid rafts modifying TSH-TSHR interaction.

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PLOS ONE

A Firmer Understanding of the Effect of Hypergravity on Thyroid Tissue: Cholesterol and Thyrotropin Receptor

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Abstract

Maintaining a good health requires the maintenance of a body homeostasis which largely depends on correct functioning of thyroid gland. The cells of the thyroid tissue are strongly sensitive to hypogravity, as already proven in mice after returning to the earth from long-term space missions. Here we studied whether hypergravity may be used to counteract the physiological deconditioning of long-duration spaceflight. We investigated the influence of hypergravity on key lipids and proteins involved in thyroid tissue function. We quantified cholesterol (CHO) and different species of sphingomyelin (SM) and ceramide, analysed thyrotropin (TSH) related molecules such as thyrotropin-receptor (TSHR), cAMP, Caveolin-1 and molecule signalling such as Signal transducer and activator of transcription-3 (STAT3). The hypergravity treatment resulted in the upregulation of the TSHR and Caveolin-1 and downregulation of STAT3 without changes of cAMP. TSHR lost its specific localization and spread throughout the cell membrane; TSH treatment facilitated the shedding of α subunit of TSHR and its releasing into the extracellular space. No specific variations were observed for each species of SM and ceramide. Importantly, the level of CHO was strongly reduced. In conclusion, hypergravity conditions induce change in CHO and TSHR of thyroid gland. The possibility that lipid rafts are strongly perturbed by hypergravity-induced CHO depletion by influencing TSH-TSHR interaction was discussed.

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Introduction

Life on earth has been and is constantly evolving for the variations of different environmental factors, with the exception of the gravitational force that has remained constant over time. The 1gravity(1 g) is responsible for the architecture and function of the cells of all living organisms. So its variation could determine unique and novel effects on cells both *in vitro* and *in vivo* with changes in their biological behavior. Scientific research is mainly devoted to study the effects of microgravity during parabolic flights and longer missions in Space on board the ISS. Nevertheless parabolic flights and spaceflight in general were accompanied by transient hypergravity and vibration [1]. During the initial launch phase, hypergravity forces due to the rocket acceleration were accompanied by launch vibration [1]. In long mission in Space, hypergravity should not be an important factor whereas a certain level of vibration originating from the different machines as well as from the astronauts themselves, for example during workout, was present [2]. Differently, during the parabolic flight each of the 31 parabolas normally flown included 22 s of microgravity and periods of normal and hypergravity [3,4]. Therefore, the impact of hypergravity on biological systems needs to be considered. In hypergravity conditions plants developed changes in orientation of cortical microtubules and in the metabolism of anti-gravitational cell wall polysaccharides with the modification of body shape and the regulation of cell wall rigidity [5]. Glycoprotein Ib-alpha surface expression and its association with the cytoskeleton were significantly increased in hypergravity-exposed platelets with the increase of their function [6]. In human T cells, ground-based studies to investigate the effect of hypergravity (1.8 g and 9 g) did not reveal any effect on cell cycle control signaling [1]. In myoblast, hypergravity stimulated both proliferation and differentiation [7]. It is widely known that musculoskeletal system together to cardiovascular, immune and nervous systems are functionally controlled by the thyroid gland. In follicular thyroid cells mRNA concentrations of growth factors varied significantly under the influence of the mechanical forces generated by hypergravity and vibration [2]. Particularly, in thyroid cells cultured *in vitro* as monolayers IL6 gene activation was very sensitive to physical forces [2]. Changes in the endocrine system and specifically in the thyroid gland represented the main human response to space-flights. We have recently shown structural/functional modifications of thyroid glands isolated from space-flight mice compared to those isolated from control laboratory-kept mice. After long-term exposure to real microgravity environment the thyroid tissues presented a more homogenous structure with prevalence of ordered and large follicles in which thyrocyte cells were thicker with bigger nuclei [8]. Both basal and

thyrotropin (TSH)-stimulated cAMP production were higher, while thyrotropin receptor (TSHR) and caveolin-1 were overexpressed [8]. Sphingomyelinase (SMase) and sphingomyelin-synthase (SM-synthase), enzymes involved in cell signaling, were equally overexpressed [9]. It seems that hypogravity and hypergravity would generate opposite results on thyroid gland, but some key results of our previous hypogravity studies were surprisingly similar. In hypergravity the SMase expression did change as in hyporgravity but the nucleus-cytoplasm translocation was similar in the two experimental conditions, suggesting that alteration of gravity conditions might be responsible for molecular remodellings which might influence the cell fate [9]. In addition, both hypogravity and hypergravity induced loss of the parafollicular cells within the thyroid gland, with consequent reduction of calcitonin production, suggesting a potential implication of the mechanical forces in the regulation of bone homeostasis via thyroid gland [10].

Results reported in the literature showed that the effects of hypergravity on the thyroid gland were only partly similar to those produced by hypogravity. In this study we investigated the influence of complex mechanical effects of hypergravity on key proteins and lipids of signal transduction in thyroid gland *in vivo*.

Materials and Methods

Reagent

Anti- Signal transducer and activator of transcription-3 (STAT3), anti-TSHR, anti-Caveolin 1, fluorescein isothiocyanate (FITC)-conjugated secondary antibody were obtained from Santa Cruz Biotechnology, Inc. (California, USA); SDS-PAGE molecular weight standards were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Chemiluminescence kits was purchased from Amersham (Rainham, Essex, UK). Cholesterol (CHO), TSH and cAMP EIA kits were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and CABRU SAS (Milan, Italy), respectively. Sphingomyelin (SM) 18:1 12:0, SM 18:1 16:0, SM 18:1 18:1, SM 24:0, phosphatidylcholine (PC) 16:0 18:1, PC 16:0 20:4, PC 18:1 18:0, ceramide 18:1 16:0, ceramide 18:1 18:0, ceramide 18:1 20:0, ceramide 18:1 24:0 were purchased from Avanti (Avanti Polar, Alabaster, USA).

Experimental design and animal care

All experimental procedures were authorized by the Public Veterinary Health Department of the Italian Ministry of Health. The experiment was also conducted in accordance with the regulations for the care and use of laboratory animals and with the guidelines of the Japanese Physiological Society. Furthermore, this study was also approved by the Committee on Animal Care and Use at Graduate School of Medicine, Osaka University (No. 22-071). Finally, the protocol utilized in the study has been authorized by the Public Veterinary Health Department of the Italian Ministry of Health. All experiments were carried out using male C57BL/10J mice (8 weeks old).

Seven mice were maintained in hypergravity in a 2×g centrifuge (2 gravity samples, 2 g) for 90 days in the laboratory of Dr. Y. Ohira at the Osaka University, Osaka, Japan. Six mice of same strain, treated with the same diet and under the same environmental conditions were maintained at the Vivarium (V samples) of the Advanced Biotechnology Center in Genova, Italy, as control samples. The control mice were similar to those previously used for hypogravity study [9]. Thyroids were sampled bilaterally from each mouse killed by inhalation of carbon dioxide and either processed or frozen immediately, according to the various experimental protocols.

Thyroid tissue treatment

After excision, right thyroid lobes of all mice under study were used to detect the effect of TSH stimulation on cAMP and TSHR amount. Left lobes of four 2 g mice and three V mice were used for UFLC-MS/MS analysis and left lobes of three 2 g and V mice were used for immunofluorescence analysis.

TSH stimulation

Right thyroid lobes were divided into 3 fragments: two fragments were treated with 10^{-7} and 10^{-8} M TSH for 1 hour, the untreated fragment was used as control. After stimulation the fragments were fixed with absolute ethanol for 10 min at room temperature and centrifuged for 20 min at 3000×g. The supernatants were used for the evaluation of cAMP levels. At this end the Cayman's cAMP assay Kit, a competitive EIA that permits cAMP measurements within the standard curve range of 0.08–10 pmol/ml, was used as previously reported [11]. The 96-well plates ready to use were supplied in the kit. Each sample was assayed in duplicate at two different dilutions for three times. 50 µl of standard or sample was added to each well. In following steps, performed according to the manufacturing instructions, was first added cAMP acetylcholinesterase (AChE), that is able to bind specific antibodies in inversely proportional way to the free cAMP do dose in the sample, and then was added Ellman's reagent containing the substrate for AChE. The product of the reaction appeared yellow and the intensity of color was measured spectrophotometrically at 412 nm.

The pellets were used to quantify protein amount and for immunoblotting analysis.

Pellet treatment

The pellets obtained after centrifugation reported in the cAMP assay were homogenized with a dounce homogenizer maintaining temperature at 4°C throughout all procedures. The suspension was used in part for protein dosage [12], in part for immunoblotting analysis by using FRTL-5 cells as potitive control.

Western immunoblotting

About 30 µg of pellet proteins were submitted to SDS-PAGE electrophoresis in 10% polyacrylamide slab gel for TSHR and STAT3 and 12% slab gel for Caveolin-1 detection. Electrophoresis image analysis was performed on gels stained with Coomassie-blue. Proteins were transferred into nitrocellulose for 90 min as previously described [13]. The membranes were blocked for 30 min with 0.5% no-fat dry milk in PBS (pH 7.5) and incubated overnight at 4°C with the anti-TSHR, STAT3, Caveolin1 specific antibody diluted 1:1000 with 0.5% no-fat dry milk in PBS. The blots were treated with HRP-conjugated secondary antibodies for 90 min. Visualization was performed with the enhanced chemiluminescence kit. The apparent molecular weight of the proteins was calculated according to the migration of molecular size standards. The area density of the bands was evaluated by densitometry scanning and analysed with Scion Image.

Lipid extraction

Lipid extraction was performed according to Matyash *et al.* [14]. Pellets of NFL and N were diluted with 1 mL methanol. 3 mL

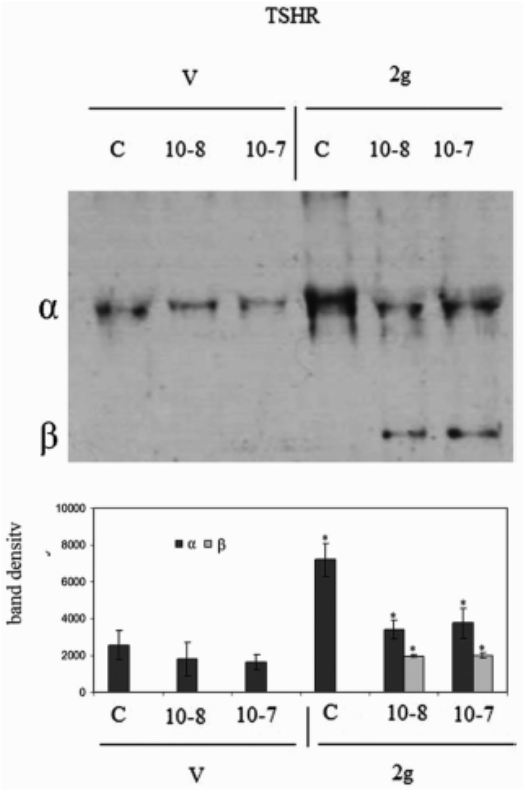

Figure 1. Effect of thyrotropin stimulation on thyrotropin receptor in hypergravity. Thyrotropin receptor in the thyroid tissue of vivarium (V) and 2 gravity (2 g) mice treated or not with 10^{-7} or 10^{-8} thyrotropin (TSH). a) 53 kDa apparent molecular weight of α subunity and 30 kDa of β subunity was indicated in relation to the position of molecular size standard; b) The area density was evaluated by densitometry scanning and analysed with Scion Image programme. The experiment was performed in right lobes of thyroid glands. Data represent the mean ± S.D. of 7 2 g mice and 6 V mice. Significance, *P<0.001 2 g versus V
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ultra pure water and 3 mL MTBE were added. Each Sample was vortexed for 1 min and centrifuged at 3000 g for 5 min. The supernatant was recovered. The extraction with MTBE was repeated on the pellet and the supernatant was added to the first. The organic phase was dried under nitrogen flow and resuspended in 500 µL of methanol.

Ultra Fast Liquid chromatography tandem mass spectrometry (UFLC-MS/MS)

Lipid standards SM 18:1 12:0, SM 18:1 16:0, SM 18:1 SM 18:1, PC 16:0 18:1, PC 16:0 20:4, PC 18:1 18:0, ceramide 18:1 16:0, ceramide 18:1 18:0, ceramide 18:1 20:0, ceramide 18:1 24:0; and CHO were prepared according to Matyash *et al.*, [14]. Standards were dissolved in chloroform/methanol (9:1 v/v) at 10 µg/mL final concentration. The stock solutions were stored at −20°C. Working calibrators were prepared by diluting stock solutions with methanol to 500:0, 250:0, 100:0, 50:0 ng/ml final concentrations.

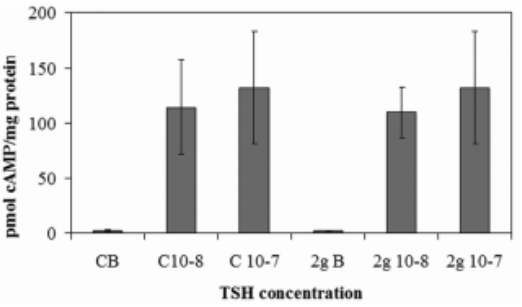

Figure 2. Effect of thyrotropin stimulation on cAMP in hypergravity. cAMP level after 10^{-7} or 10^{-8} thyrotropin (TSH) stimulation in hypergravity. The results were compared with those obtained in vivarium. The experiment was performed in right lobes of thyroid glands The data were expressed as pmol/mg protein and represent the mean ± S.D. of 2gravity (2 g) mice and 6 vivarium (V) mice.
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20 µL of standards or lipids extracted from NRL or N samples were injected after purification with specific filter in nylon (0.2 µm).

Analyses were carried out according to Rabagny *et al.* [15] by using Ultra Performance Liquid Chromatography system tandem Mass Spectometer Applaied biosistem (Shimadzu Italy s.r.l., Italy). The lipid species were separated, identified and analysed by following the methods of Rabagny *et al.* [15].

Immunofluorescence analysis

Tiroid tissues were fixed in 4% neutral phosphate-buffered formaldehyde solution for 24 h. Lobes were dropped with essentially random orientation in paraffin. The paraffin blocks were sectioned into 4-µm-thick sections. All sections were mounted on silan-coated glass slides. Each slide contained a pair of sections at a distance equal to 140 µm. Between 7 and 14 pairs of sections were previously used [9,10]. In the present study between 15 and 20 pairs of sections were sampled and 17, 18, 19 sections were used for immunofluorescence analysis.

Tissue sections were deparaffinized and rehydrated through a series of xylene and ethanol washes. After 3 washes with phosphate-buffered saline (PBS), sections were incubated with 2 µg/ml anti TSHR primary antibodies diluted in a 0.5% solution of bovine serum albumin in PBS overnight at 4°C. The slides were washed 3 times with PBS and incubated with FITC fluorochrome-conjugated secondary antibodies for 1 hour at room temperature. After 3 washes with PBS, the slides were mounted with glycerol and coverslips. The samples were examined under a fluorescence microscope (OLYMPUS IX 51) equipped with an OLYMPUS DP 50 camera system and analyzed at 20x magnification.

Statistical analysis. Data were expressed as means ± SD and their significance was checked by Student 's t-test. P<0.01 versus control samples.

Results

1. Hypergravity up-regulates thyrotropin receptor surface protein but the response to hormonal treatment remains unchanged

Experiments of immunoblotting demonstrated that the exposure to hypergravity increased TSHR content (Fig.1a). In the present study the

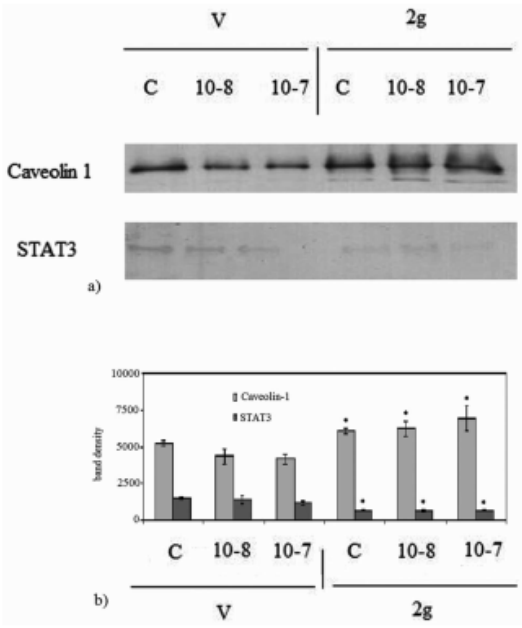

Figure 5. Effect of hypergravity on Caveolin 1 and STAT3 proteins. Caveolin 1 and STAT3 in the thyroid tissue of vivarium (V) and 2 gravity (2 g) mice treated or not with 10^{-7} or 10^{-8} thyrotropin (TSH). a) bands of 22 kDa apparent molecular weight for Caveolin 1 and 90 kDa for STAT3 was indicated in relation to the position of molecular size standard; b) The area density was evaluated by densitometry scanning and analysed with Scion Image programme. The experiment was performed in right lobes of thyroid gland Data represent the mean ± S.D. of 7 2 g mice and 6 V mice. Significance, *P<0.001 2 g versus V.
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immunoblotting was performed in the pellet obtained with centrifugation after fixation of tissue fragments with absolute ethanol, treatment useful for cAMP evaluation. In the control (C) of both V and 2 g samples we obtained only a single band corresponding to 53 kDa apparent molecular weight (α subunit). The density of the 2 g C band was 2.8 times higher than that of the V C (Fig.1b). 10^{-7} or 10^{-8} TSH treatment did not cause significant changes in the thyroid gland of the V mice (Fig.1a). In contrast, the same treatment in thyroids of mice subjected to 2 g induced about 50% reduction of the α subunit and the appearance of the β subunit, corresponding to 30 kDa apparent molecular weight (Fig.1a, 1b). Thus we have tested TSH-stimulated cAMP production to verify the sensitivity of the thyroid cells to hormone in hypergravity conditions. The results showed a similar basal and TSH-stimulated cAMP production in V and 2 g samples, indicating that even if the TSHR was up-regulated in hypergravity, the loss of its α subunit that links TSH was responsible for the lack of the expected response to hormonal stimulation (Fig.2). It is possible that hypergravity induces a membrane remodelling so that the TSH treatment might facilitate an easier extraction of the β subunit, despite the ethanol treatment. It is therefore possible that perception of hypergravity from the cell membrane may be the first step in the cell response. To verify this hypothesis we performed immunofluorescence analysis of TSHR with specific antibody. The results demonstrated that in V sample the receptor was present on the surface of thyrocytes that surrounded follicles with a precise location, as shown by the sharp brightness of the fluorescent signal (Fig.3). In 2 g samples the fluorescence levels were higher, supporting the data obtained with immunoglotting. The fluorescent signal was also spread over the entire surface of thyrocytes (Fig.3). Since TSHRs were G protein-coupled receptors [16] which were localized in membrane microdomains, including caveolae and lipid rafts enriched in SM and CHO content [17,18], we next considered if the distribution of TSHR over the entire cell surface in hypergravity conditions could be due to the alteration of lipid component of cell membrane. Thus we performed a UFLC-MS/MS study on lipid fraction of thyroid tissue focusing our attention on SM species and CHO. We have first analyzed the amount of specific species of saturated and mono-unsaturated fatty acids SM, PC and ceramide in order to establish possible specific variation of SM species by using specific standards. Our results showed that not only PC and ceramide species, but also SM species remained unchanged (Fig.4a). Differently, the level of CHO reduced 49% (Fig.4b). Considering the relationship SM-CHO known in the literature, we extended our observation to the SM species containing longer fatty acid chains and with greater degrees of unsaturation. The identification of the molecules was carried out according to the RT and the molecular weight. The comparison between V and 2 g samples was carried out by calculating the percentage of the areas. No significant changes were found for each SM species (Fig.4c) and for saturated/unsaturated ratio of total SM (Fig.4 d).

2. Gravity controls structural/functional proteins

To explore the mechanisms underlying the effect of gravity on thyroid protein fraction, we examined proteins relevant for the thyroid function; Caveolin-1 which is a critical protein to caveolae and STAT3 which is a suppressor of thyroid tumor growth [19]. Our results showed the increase of Caveolin-1 protein in both TSH-untreated and TSH treated thyroid tissues of 2 g mice in comparison with V mice (Fig. 5a). In fact, the density value of Caveolin-1 band, corresponding to 22 kDa apparent molecular weight, was 14% higher in 2 g C than V C and it did not change significantly after TSH treatment (Fig.5b). Immunopositivity of STAT3 was reduced in 2 g mice in comparison with V mice (Fig.5a). The area density of STAT3 protein, corresponding to 90 kDa apparent molecular weight, was 53% lower in 2 g C than V C and it unchanged significantly after TSH treatment (Fig.5b).

Discussion

Based on our previous results of hypergravity-challenged thyroid glands [9,10] we had hypothesized that under hypergravity conditions thyrocytes might remodel their cell membrane by increasing TSHR surface protein and consequently change their sensitivity to TSH treatment. Our results showed that this relationship is not so straightforward. TSHR is a G protein-coupled receptor [16]. It is a heterodimer [20] that includes an α extracellular subunit (53 kDa), that interacts with TSH, and a broad β transmembrane and intracellular subunit (30–42 kDa), held together by disulphide bridges [21]. Costa *et al.* [22] demonstrated that TSHRs were not distributed randomly in cell membranes but were rather localized in the basolateral membrane rich in lipid rafts, microdomains with high level of CHO and sphingolipids [23]. Differently, Latif *et al.* [24] demonstrated that monomeric and multimeric TSHRs were present in cell surface in both raft and no-raft fractions as caveolae. However the raft domains provided a platform for the assembly of signaling complexes of receptors [25]. Our results showed that hypergravity up-regulated 65% TSHRs and only 14% Caveolin-1. In addition, treatment with TSH in 2 g samples strongly changed the TSHR but had no effect on Caveolin-1. We thus suggest that gravity forces may remodel

cell membrane structure by acting specifically on lipid rafts. Loosfelt *et al.* [20] indicated that TSH treatment facilitated the shedding of α subunit of TSHR and its releasing into the extracellular space. We demonstrated that TSH treatment did not induce a significant reduction of the TSHR α subunit in V mice, whereas it was reduced 2.1 times in 2 g mice. In C V mice, after TSHR treatment we would have expected two bands of α and β subunits and an evident reduction of the α subunit. It is useful to consider in this regard that we have carried out the evaluation of the TSHR in the pellet obtained after treatment of the tissue with ethanol necessary for the analysis of the production of cAMP. So it is possible that the ethanol had a fixative effect on the membranes by impairing the shedding of TSHR α subunit after TSH treatment and the extraction of the intramembrane β subunit. The behavior of TSH subunits in hypergravity samples was very different. Based on these findings, our opinion was that lipid rafts were strongly perturbed by hypergravity-induced CHO depletion, so that the TSHR lost its specific localization and spread throughout the cell membrane, as demonstrated by immunofluorescence. It was likely that the spatial relationship of the two subunits could be amended during the movement in such a way that the treatment with TSH could induce shedding of α subunit and extraction of β subunit, despite treatment with ethanol. The results presented in this paper have been really intriguing to us because only two years ago we obtained similar results on thyrocytes cultured under conditions of microgravity [11]. In that experiment FRTL-5 thyroid cell line was exposed to microgravity during the Texus-44 mission. In such circumstance, our *in vitro* system had allowed us to demonstrate the presence of the TSHR and the increase of CHO in the culture medium. In hypogravity the cAMP production after TSH stimulation was inhibited with respect to control samples [11]. Here instead we observed similar results in hypergravity and in control samples. Such difference could be due to the upregulation of the TSHR in hypergravity. Microgravity also induced the release of total SM measured by thin layer chromatography. Here with a more sophisticated technique we did not notice any significant variation of individual SM species. On the other hand we had already shown that the enzymes responsible for SM metabolism behaved similarly under hypo- and hypergravity conditions [9]. In hypergravity, no variation of SMase and SM-synthase1 expression was found as in hypogravity but the SMase translocated from the nucleus to the cytoplasm and had similar values of enzyme activity to those in hypogravity [9]. Comparing the results from hypo-and hypergravity, it was evident that the plasma membrane was significantly altered either increasing or decreasing the mechanical load of the gravitational force. CHO removal perturbed lipid rafts that act as platform for TSHR. Therefore membrane CHO and not SM was critical for TSH-TSHR interaction. Depletion of membrane CHO by methyl-beta-cyclodextrin resulted in a disruption of lipid rafts in plasma membrane [26] whereas extensive sphingolipid depletion did not affect lipid raft integrity [27]. Rapid changes of the cytoskeleton as reaction to gravitational changes were reported in diverse cell types [28,29] and they could be responsible for changes in surface receptors [1]. Here we reported the first observation of altered receptor by perturbation of membrane CHO.

Author Contributions

Conceived and designed the experiments: EA FC FSAI. Performed the experiments: AL AF RL SC EL IF. Analyzed the data: EA FC. Contributed reagents/materials/analysis tools: FSAI FC. Wrote the paper: EA FSAI.

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COMMENTO DEL VII° LAVORO

I risultati hanno dimostrato che l'ipergravità induce una over-espressione del TSHr pari al 65%. Il trattamento con TSH induce una riduzione di circa il 50% della subunità alfa del TSHr.

La produzione di AMPc successive alla stimolazione con TSH è simile nel campione controlllo e nel campione in ipergravità, anche se il TSHr è over-espresso la perdita della sua sub-unità alfa è responsabile del mancato aumento della risposta alla stimolazione ormonale.

L'immunoflorescenza ha dimostrato che il TSHr nei campioni controllo si localizza sulla superficie dei tireociti in modo specifico mentre nei campioni in ipergravità il segnale fluorescente è più forte ed è diffuso su tutta la superficie dei tireociti. Lo studio con UFLC-MS/MS sulla frazione lipidica del tessuto tiroideo ha mostrato che le Specie di SM e Ceramide erano invariate rispetto hai campioni controllo mentre il CHO era ridotto del 49%.

8- INTRODUZIONE AL VIII° LAVORO

É noto che I fasci di protoni inibiscono la crescita cellulare con un blocco della fase G1/G0 e G2/M del ciclo cellulare, inducono aberrazioni cromosomiche e apoptosi in cellule di melanoma.

Poichè non vi erano dati in letteratura sull'effetto di fasci di protoni in cellule tiroidee e poichè noi avevamo precedentemente dimostrato che le radiazioni UV-c inducevano danni sui tireociti, nel presente lavoro abbiamo studiato l'effetto dei protoni sui tireociti in coltura della linea FRTL-5 come riportato in materiali e metodi. In particolare abbiamo analizzato il comportamento del metabolismo della SM in lisati liberi dai nuclei e in nuclei purificati in cellule tiroidee a diverso stato funzionale, proliferanti o quiescenti.

Title: UFLC MS/MS to Identify the Response to Proton Radiations of FRTL-5 cells.

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Purpose: In cell nucleus the sphingomyelin (SM) cycle is regulated by an exact internal mechanism. In no-stimulated cells exist a balance between neutral-sphingomyelinase (N-SMase) and sphingomyelin-synthase (SM-synthase) activity that maintains constant the SM level, stabilizing the double helix of DNA thanks to the trimethylammonio group that binds to the phosphate group of DNA and apolar fatty acids that bind to the hydrophobic centres of the helical DNA. The increase of the nuclear SM concentration during cell proliferation stimulates the SMase activity during the S phase of the cell cycle, thus favouring the destabilization and the opening of the double helix of DNA and the beginning of its synthesis. The winding of a double helix being restored as soon as DNA synthesis finishes and SM-synthase activity is stimulated. During apoptosis specific the gene expression and DNA fragmentation are accompanied by the change of nuclear SM content. Recently the nuclear N-SMase/SM-synthase ratio was considered a marker of cell function. In fact it was very high in apoptotic cells, medium in pro-apoptotic cells, low in proliferating cells and very low in quiescent cells. Here we studied for the first time whether in thyroid FRTL-5 cells the proton beams could change nuclear SM metabolism by comparing the results with that occurred in nuclei-free lysates (NFL) at the end to identify their role on functional state of the cells.

Results: In the nucleus proton beams stimulated 1.09 times N-SMase activity and inhibited 1.9 times that of SM-synthase in nuclei purified from quiescent cells whereas stimulated 12.44 times N-SMase activity and unchanged that of SM-synthase. The effect was very low in NFL lipid enzymes. As a consequence, the nuclear N-SMase/SM-synthase ratio was 1.06 and 1.77 in nuclei of quiescent and proliferating cells respectively, indicating that protons induced quiescent cells in a propaoptotic state whereas its value in proliferating cells was between the proapoptotic state and apoptotic state, as supported by bax expression that was higher in proliferating than in quiescent cells. The analysis of nuclear SM and ceramides by UFLC-MS/MS showed the variation of these lipids after proton treatment.

Conclusion: The damage of proton beams on epithelial thyroid cells depends on their physiological state, whether they are stimulated or not with thyrotropin to proliferate. The nuclear SM metabolism is involved in the mechanism of action of protons.

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Article

Critical Role for the Protons in FRTL-5 Thyroid Cells: Nuclear Sphingomyelinase Induced-Damage

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Abstract: Proliferating thyroid cells are more sensitive to UV-C radiations than quiescent cells. The effect is mediated by nuclear phosphatidylcholine and sphingomyelin metabolism. It was demonstrated that proton beams arrest cell growth and stimulate apoptosis but until now there have been no indications in the literature about their possible mechanism of action. Here we studied the effect of protons on FRTL-5 cells in culture. We showed that proton beams stimulate slightly nuclear neutral sphingomyelinase activity and inhibit nuclear sphingomyelin-synthase activity in quiescent cells whereas stimulate strongly nuclear neutral sphingomyelinase activity and do not change nuclear sphingomyelin-synthase activity in proliferating cells. The study of neutral sphingomyelinase/sphingomyelin-synthase ratio, a marker of functional state of the cells, indicated that proton beams induce FRTL-5 cells in a proapoptotic state if the cells are quiescent and in an initial apoptotic state if the cells are proliferating. The changes of cell life are accompanied by a decrease of nuclear sphingomyelin and increase of bax protein.

Keywords: protons; thyroid cells; sphingomyelin; neutral-sphingomyelinase; apoptosis; sphingomyelin-synthase

1. Introduction

Proton beams induced chromosomal aberrations [1,2]. The relative biological effectiveness was both dose- and depth-dependent [3]. In HTB63 human melanoma cells, proton beams inhibited cell growth with G2/M and G1/G0 arrest of the cell cycle and appearance of apoptotic nuclei, even 48 h after irradiation [4].

To date we have very little information on the effect of protons on the endocrine system. Just a paper exists about the thyroid, an endocrine gland that regulates the metabolism of cardiovascular, musculoskeletal, immune and nervous system by influencing body equilibrium [5]. The authors showed that the sensitivity of thyroid follicular cells (FRTL-5 cell line) to proton irradiation was independent of their ability to communicate through connexin 32 gap junctions, but there are no indications in the literature on their possible mechanism of action. We have previously demonstrated that UV-C radiation induced apoptosis of FRTL-5 cells, by changing nuclear lipid metabolism in relation to the physiological state of cells [6]. We used FRTL-5 cells because *in vitro* they permanently express most of *in vivo* tissue-specific thyroid characteristics, such as thyroglobulin synthesis and secretion, iodide active transport, peroxidase production and thyrotropin (TSH) sensitivity [7]. It is known that TSH treatment induced thyroid cells to the proliferative state whereas TSH starvation rendered them quiescent [8]. Proliferating cells were more sensitive to UV-C radiation treatment than quiescent cells by changing phosphatidylcholine (PC) and sphingomyelin (SM) metabolism, specially in lipid localised at nuclear level. In nuclei purified from proliferating cells, irradiation stimulated neutral-sphingomyelinase (*N*-SMase) activity and inhibited sphingomyelin-synthase (SM-synthase) and phosphatidylcholine-specific phospholipase C (PC-PLC) with the consequent increase in the ceramide/diacylglycerol ratio. This effect was low in quiescent cell nuclei. The results suggested that quiescent FRTL-5 cells were more resistant to the effects of UV-C radiation because their nuclear PC and SM metabolism was less modified than that of proliferating cells [6]. In the stratosphere, depletion of ozone resulted in an enrichment of UV radiation content and global warming. It is interesting to notice that in a space environment the FRTL-5 cell changes occurred according to their physiological state, the effect being stronger in proliferating than in quiescent cells [9]. Cells did not present signs of DNA fragmentation characteristic of apoptotic process but, in contrast, showed strongly altered lipid metabolism with activation of nuclear *N*-SMase and inhibition of PC-PLC and SM-synthase [8]. The spaceflight impaired cell growth of FRTL-5 exposed to TSH; thus, the cells became similar to quiescent cells with similar SMase and SM-synthase activities. More precisely the space environment induced the cells into a pro-apoptotic state, similar to that obtained with both TSH starvation and serum withdrawal [9]. Comparing the results in different experimental models [6,9], we demonstrated that the SMase/SM-synthase ratio changed in various functional states of the cells since its value was very high in apoptotic cells, medium in pro-apoptotic cells, low in proliferating cells and very low in quiescent cells. This parameter has been suggested to be a specific marker for cell function [9]. In fact,

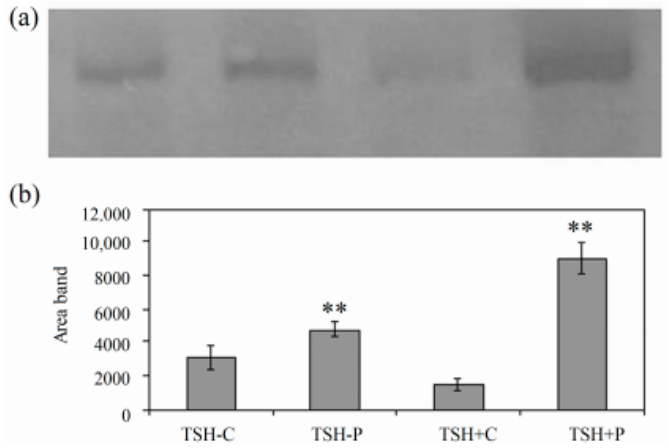
its value is low in quiescent cells and gradually increases in proliferating, pro-apoptotic and apoptotic cells [9]. Since thyroid cells are particularly sensitive to UV-C and space environment and there are no data in the literature about the effects of protons on these cells, here we studied for the first time if the proton beams could change SM metabolism in nuclei-free lysates (NFL) and/or in purified nuclei (N) prepared from thyroid FRTL-5 at the end to identify their role on functional state of the cells.

2. Results and Discussion

2.1. Proton Beams Promote Bax Expression in FRTL-5 Cells in Relation to Their Physiological State

It has been demonstrated that proton beams inhibited cell growth and induced apoptosis in melanoma cells [2]. To demonstrate if the effect of protons was specific for proliferating cells, such as tumor cells or others, we used an experimental model in which the same cells can be maintained in quiescent or proliferating state. Thyroid FTRL-5 cells were cultured in the presence of glycil-L-histidyl-L-lysine acetate, hydrocortisone, insulin, somatostatin, transferrin without (TSH−) or with TSH (TSH+). As previously reported, TSH+ cells are proliferating and TSH− cells are quiescent cells [6]. Here we showed that proton beams increased expression of Bax protein specifically in proliferating cells (Figure 1). The Bax protein analysis actually showed a stronger immunopositivity in irradiated cells in comparison to control samples (Figure 1a). Bax protein levels increased approximately 1.6 and 6.2 fold, respectively in TSH− and TSH+ cells exposed to proton irradiation, compared to unirradiated quiescent and proliferating controls (Figure 1b). The changes of Bax protein levels were evaluated by the analysis of the area of immunoblotting bands as previously reported [10].

Figure 1. Bax analysis in quiescent control sample (TSH−C) and quiescent proton-irradiated sample (TSH−P), in proliferating control sample (TSH+C) and proliferating proton-irradiated sample (TSH+P). C, control; P, proton. (**a**) Immunoblots of proteins (30 μg) were probed with anti-Bax antibodies (apparent molecular weight 23 kDa) and visualized by ECL; (**b**) The area density was evaluated by densitometry scanning and analysed with Scion Image; data represent the means ± S.D. of four separate experiments. irradiated samples significance ** $p < 0.01$ *versus* control.



2.2. Proton Beams Act on Nuclear Sphingomyelin Metabolism

In both NFL and N fractions *N*-SMase activity was higher in proliferating than in quiescent cells whereas SM-synthase activity did not change significantly with cell state (Figure 2a,b), supporting previous results [6]. In NFL sample, protons increased *N*-SMase activity 1.45 and 1.52 times in quiescent and proliferating cells, respectively (Figure 2a). In addition, while *N*-SMase activity increased only 1.09 times in N purified from quiescent cells, in N purified from proliferating cells its value increased 12.44 times (Figure 2b). Irradiation unchanged SM-synthase activity in NFL prepared from both cells and in N from proliferating cells whereas it was inhibited 1.9 times in N purified from quiescent cells (Figure 2a,b).

As a consequence, *N*-SMase/SM-synthase ratio, was 8.6 in NFL and 1.77 in N prepared from proliferating cells whereas its value was 6 in NFL and 1.06 in N prepared from quiescent cells (Figure 3), similar to that previously reported [9]. The proton treatment increased the value of *N*-SMase/SM-synthase ratio to 11.75 and 8.46 in NFL and N respectively in quiescent cells and to 11.39 and 22.97 in NFL and N respectively (Figure 3). Thus, the effect of protons was stronger in nuclear fraction than in NFL.

Figure 2. Effect of proton irradiation on neutral-sphingomyelinase (*N*-SMase) and sphingomyelin-synthase (SM-synthase) activity in: (**a**) nuclei-free lysates (NFL) and (**b**) purified nuclei (N). C, control; P, proton. Data are expressed as pmol/mg protein/min and represent the mean and range of three separated experiments.

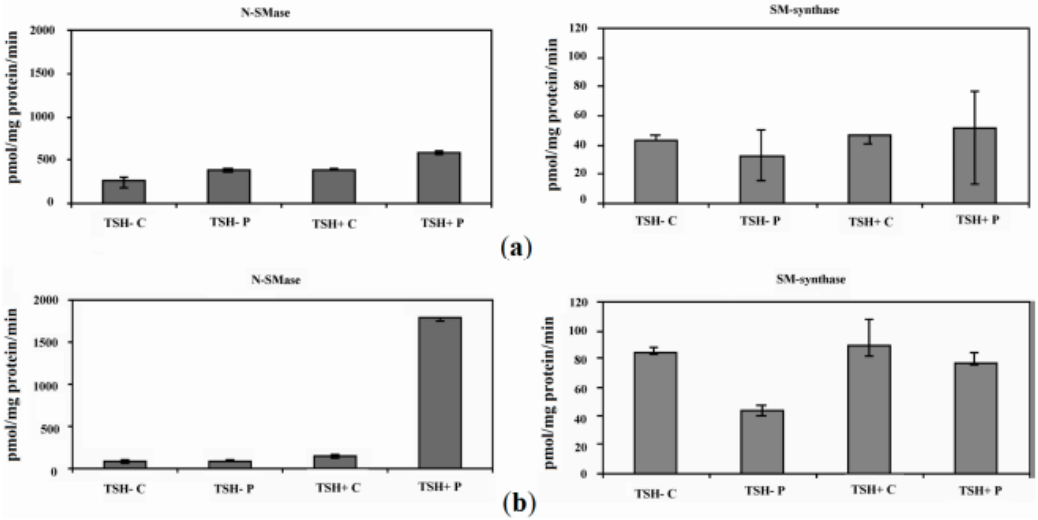
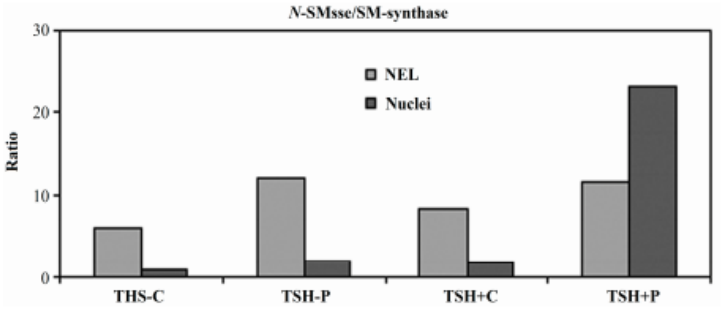


Figure 3. Effect of proton irradiation on neutral-sphingomyelinase/sphingomyelin-synthase (*N*-SMase/SM-synthase) ratio of nuclei-free lysates (NFL) and purified nuclei (N) prepared from quiescent (TSH−) and proliferating (TSH+) cells. C, control; P, proton.



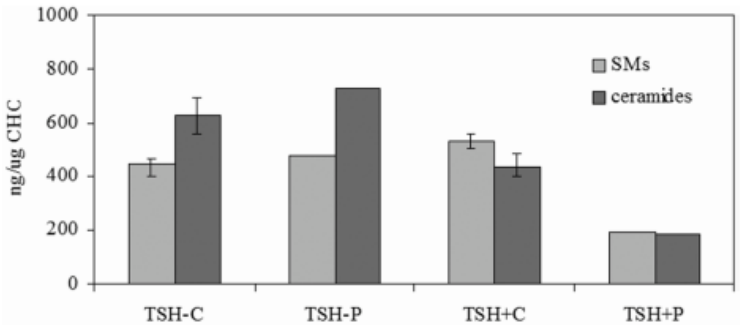
The *N*-SMase/SM-synthase ratio has been proposed to be a specific marker for cell function [9]. Its value was 8.16 ± 1.00, 5.77 ± 0.60, 14.00 ± 0.9 and 36.73 ± 3.30 in NFL and 1.77 ± 0.2, 1.06 ± 0.10, 5.39 ± 0.8, 47.80 ± 5.2 in N prepared from proliferating, quiescent, proapoptotic and apoptotic cells respectively [8]. Therefore our results indicated that protons induced quiescent cells in a propaoptotic state as well as occurred for proliferating cells if you considered the change of *N*-SMase/SM-synthase ratio in NFL. Otherwise, if you considered *N*-SMase/SM-synthase ratio in purified N, its value was between the proapoptotic state and apoptotic state, suggesting that the metabolism of the nuclear SM was more sensitive than that of NFL and probably it was the first that changed when the cells were induced to apoptosis. It is really hard to indicate exactly why protons have more effects on the SM metabolism in N than in NFL. Since the activation of the nuclear SMase reduced the amount of SM localized in the inner nuclear membrane where binded to cholesterol (CHO) to acts as a platform for DNA duplication and transcription [11] is possible that protons use this method to induce apoptosis. These data supported the more expression of bax protein in proliferating cells than in quiescent cells treated with protons.

To study the effect of *N*-SMase/SM-synthase ratio on nuclear lipids, the most representative species of SM and ceramide in the N were studied and they were referred to CHO content. The results showed that the change of CHO observed in the samples was not statistically significant. In fact, CHO value was 9.18 ± 1.76 and 7.86 ± 2.20 ng/mg protein in TSH− and TSH+ control samples and 10.20 ± 1.2, and 11.20 ± 1.34 ng/mg protein in TSH− and TSH+ proton-treated samples.

The limit of this part of the present study is that we had only one sample for the SM and ceramide analysis of proton-exposed samples and we did not have the possibility to repeat the exposure at the moment. Therefore, only strong changes were considered. The N fraction of the proliferating cells was richer in SM and poorer in ceramide content than that of quiescent cells (Figure 4). The role of nuclear SM on cell proliferation has been widely demonstrated [12–14]. SM facilitated DNA stabilization [12]. The trimethylammonium group of SM bonded to the phosphate group of DNA, whereas its apolar fatty acids bonded to the hydrophobic centers of the internal part of the helical DNA, facilitating its stabilization [12]. The increase of the nuclear SM concentration during cell proliferation stimulated the SMase activity during the S phase of the cell cycle, thus favoring the initiation of DNA synthesis [15]. Our results, showing the high level of SM and SMase activity in purified N of proliferating FRTL-5

cells, confirmed previous observation obtained in liver. As *N*-SMase was more active in proliferating cells, we would expect a higher content of ceramide. Instead, the content of ceramide was reduced (Figure 4), probably being rapidly moved to the cytoplasm. The most represented species were ceramide 18:1 16:0 and ceramide 18:1 24:0 and they were reduced 1.62 and 1.48 times respectively with protons (Table 1).

Figure 4. Effect of proton irradiation on total sphingomyelin (SM) and ceramide species under study of nuclei-free lysates (NFL) and purified nuclei (N) prepared from quiescent (TSH−) and proliferating (TSH+) cells. C, control; P, proton. Data are expressed as μg/μg cholesterol (CHO) and are expressed as mean and range of three separated experiments for C samples. Results of P samples are only of one experiment.



The proton beams treatment of quiescent cells induced a low increase of total ceramide (Figure 4) due probably to the SM-synthase inhibition more than to the low increase of *N*-SMase activity (Figure 2). In particular, ceramide 18:1 20:0 increased (Table 1). In proliferating cells treated with protons, the content of total SM was reduced 2.76 times due to the high activity of *N*-SMase (Figure 2) and the content of total ceramides was reduced 2.33 times (Figure 4). All species of ceramide were reduced with the exception of ceramide 18:1 18:0 (Table 1). We do not know at the moment if the reduction of ceramides could be due to the nucleus-cytoplasm translocation or to the presence of a ceramidase enzyme in the N fraction of FTRL-5 cells, which however has not been demonstrated until now.

Table 1. Species of sphingomyelin (SM) and ceramide in nuclei of FRTL-5 cells. Data of proton (P) are expressed as ng/μg cholesterol and represent the mean of two samples very similar. TSH−, quiescent cells; TSH+, proliferating cells; C, control; P, proton. Data of control (C) are expressed as mean and range of three independent experiments.

SM Species	TSH−C	TSH−P	TSH+C	TSH+P
SM 18:1 12:0	0.7 (0.5–0.8)	0,9	0.3 (0.2–0.6)	0.15
SM 18:1 16:0	46 (41–58)	50	64 (55–71)	20
SM 18:1 18:1	5 (3.3–5.6)	2,2	1.4 (1.1–1.5)	1.4
SM 24:0	172 (160–203)	184	188 (168–202)	74
ceramide 18:1 16:0	117 (102–139)	147	72 (62–68)	25
ceramide 18:1 18:0	31 (23–36)	48	2 (1.6–2.3)	8
ceramide 18:1 20:0	45 (18–54)	118	62 (40–71)	27

3. Experimental Section

3.1. Reagents and Standards

PC, SM, non-hydroxy fatty acid ceramide, phenylmethylsulfonylfluoride, acetonitrile, methanol, 2-propanol, metyl-tert-butyl ether (MTBE), formic acid, chloroform, cholesterol (CHO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA.); TLC plates (silica Gel G60) were from Merck (Darmstadt, Germany); the radioactive [Me-^{14}C] SM (54.5 Ci/mol, 2.04 GBq/mmol), [Me-^{3}H] (L-3-phosphatidyl-[*N*-Me-^{3}H]-choline-1,2-dipalmitoyl, 81.0 Ci/mmol, 3.03 TBq/mmol) were from Amersham Pharmacia Biotech (Rainham, Essex, UK); Ecoscint A was from National Diagnostic (Atlanta, GA, USA.). Anti-Bax was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). SDS-PAGE Molecular Weight Standard was from Bio-Rad Laboratories (Hercules, CA, USA). SM 18:1 12:0, SM 18:1 16:0, SM 18:1 18:1, SM 24:0, ceramide 18:1 16:0, ceramide 18:1 18:0, ceramide 18:1 20:0, ceramide 18:1 24:0 were purchased from Avanti (Avanti Polar, Alabaster, AL, USA).

3.2. Cell Cultures and Treatments

FRTL-5 cells were prepared and characterized in the Ambesi-Impiombato laboratory as previously reported [9]. Cells were grown in Ham's modified F-12 with 5% calf serum and 6 hormones: 10 ng/mL glycil-L-histidyl-L-lysine acetate (Sigma), 10^{-8} M hydrocortisone (Sigma), 10 µg/mL insulin (Sigma), 10 µg/mL somatostatin (Sigma), 5 µg/mL transferrin (Sigma), 10 mU/mL TSH (Sigma). FRTL5 were maintained at 37 °C in 5% of CO_2, 95% humidity incubator. The cells cultured in the presence of TSH that stimulates cell proliferation were called TSH+ cells, whereas the cells cultured without TSH remained in the quiescent state and were called TSH− cells [6]. Cells were counted and seeded at 5×10^5/60 mm plastic dish concentration in TSH+ medium to permit adhesion. After 24 h, culture continued for 7 days in TSH+ or TSH− medium. For the experiment, proliferating and quiescent FRTL-5 cells were collected and then seeded at a density of 1×10^6 cells/1.0 cm^2 chamber with 1 mL TSH+ or TSH− medium for 24 h in incubator to permit adhesion and then each chamber placed inside boxes suitable for cultivation where the temperature was set at 37 °C. After plating, the cells were submitted to proton beams produced and accelerated by the CERN (Geneva, Switzerland) accelerators. Radio-frequency linear accelerators were used as injectors for synchrotrons and as stand-alone accelerators for the production of intense particle beams, thanks to their ability to accelerate high beam currents at high repetition rates. The cells were used in part for NFL and N preparation and in part for the analysis of Bax apoptotic protein. Both NFL, containing all cell membranes except nuclear membranes, and purified N were used to analyze the effect of proton beams on SM localized in different subcellular compartments. NFL and N were prepared as previously reported [6]. Cells were washed twice with PBS and centrifuged at 800× *g* for 10 min. The pellet was suspended in hypotonic buffer (1.5 M sucrose, 3 mM $CaCl_2$, 2 mM Mg acetate, 0.5 mM dithiothreitol, 1 mM PMSF, 3 mM Tris–HCl pH 8.0, 1 mL/10^6 cells) and gently homogenized by a tight-fitting teflon-glass homogenizer. Part of the homogenate was centrifuged at 500× *g* for 30 min at 4 °C for NFL preparation and part was used for nuclei isolation. At this end homogenized cells were treated with 1% Triton X-100 in hypotonic buffer (0.5:1 *v*/*v*); the cellular suspension was stirred on a vortex

mixer for 30 s and the buffer, containing 1.5 M sucrose, was added (0.25:1 *v*/*v*). After centrifugation at 2000× *g* for 10 min the pellet containing nuclei was washed twice with Barnes *et al.* solution [16] 0.085 M KCl, 0.0085 M NaCl, 0.0025 M $MgCl_2$, trichloroacetic acid–HCl 0.005 M, pH 7.2).

3.3. Lipid Extraction

Lipid extraction was performed according to Matyash *et al.* 2008 [17]. Pellets of NFL and N were diluted with 1 mL methanol. Exactly 3 mL ultra pure water and 3 mL MTBE were added. Each sample was vortexed for 1 min and centrifuged at 3000× *g* for 5 min. The supernatant was recovered. The extraction with MTBE was repeated on the pellet and the supernatant was added to the first. The organic phase was dried under nitrogen flow and resuspended in 500 µL of methanol.

3.4. Ultra Fast Liquid Chromatography Tandem Mass Spectrometry (UFLC-MS/MS)

Lipid standards (SM 18:1 12:0, SM 18:1 16:0, SM 18:1 SM 18:1, ceramide 18:1 16:0, ceramide 18:1 18:0, ceramide 18:1 20:0, ceramide 18:1 24:0; sphinganine 18:1; glucosyl ceramide 18:1 16:0 and CHO) were prepared according to Matyash *et al.* [17]. Standards were dissolved in chloroform/methanol (9:1 *v*/*v*) at 10 µg/mL final concentration. The stock solutions were stored at −20 °C. Working calibrators were prepared by diluting stock solutions with methanol to 500:0, 250:0, 100:0, 50:0 ng/mL final concentrations. Twenty micro liters of standards or lipids extracted from NRL or N samples were injected after purification with specific nylon filters (0.2 µm).

Analyses were carried out according to Rabagny *et al.* [18] by using Ultra Performance Liquid Chromatography system tandem Mass Spectometer Applaied biosistem (Shimadzu Italy s.r.l., Milano, Italy). The lipid species were separated, identified and analyzed by following the methods of Rabagny *et al.* [18].

3.5. Neutral-Sphingomyelinase Assay

The *N*-SMase activity was detected as previously reported [6] in NFL and in N. The reaction mixture contained 0.1 M Tris–HCl pH 7.6, 0.1 mM-^{14}C SM, 6 mM $MgCl_2$, 0.1% Triton X-100 and 100 µg protein of NFL or N to a final volume of 0.1 mL. Incubations were performed at 37 °C for 45 min. The reaction was stopped by adding 2 mL chloroform and methanol (2:1), 0.4 mL of 0.5% NaCl was added to the tubes and vortexed. After centrifugation at 2000 rpm × 10 min, the upper phase was removed and 0.5 mL was diluted in counting vials with 10 mL Ecoscint A and 1 mL distilled water; radioactivity was measured with a Packard liquid scintillation analyzer. Protein determination was performed as previously reported [6] and the enzyme activity was referred to protein content.

3.6. Sphingomyelin-Synthase Assay

The SM-synthase activity was detected as previously reported [6] in NFL and in N. The reaction mixture contained 0.1 M Tris–HCl, 0.3 mM ^{3}H-PC, 2 mM $CaCl_2$, 0.1% Triton X-100, 0.15 mM non-hydroxy fatty acid ceramide and 100 µg protein of NFL or N, to a final volume of 0.1 mL. Incubations were performed at 37 °C for 45 min. The reaction was stopped by adding 2 mL

chloroform and methanol (2:1), 0.4 mL of 0.5% NaCl was added to the tubes and vortexed. After centrifugation at 2000 rpm × 10 min, the lower phase was dried under nitrogen flow, lipids were re-suspended with chloroform and separated with thin-layer silica gel chromatography (TLC), using chloroform/methanol/ammonia (65:25:4) as solvent. In the sample, exogenous SM was added to the tubes before chromatography. SM was localized with iodine vapor, scraped into counting vials and diluted with 10 mL Ecoscint A and 1 mL water. Radioactivity was measured as reported for *N*-SMase activity.

3.7. Immunoblotting Analysis

Bax protein was evaluated with western blot analysis. About 30 μg proteins were loaded onto SDS-PAGE electrophoresis in 10% polyacrylamide slab gel and transferred into nitrocellulose in 75 min as previously reported [6]. Membranes were blocked for 30 min with 0.5% no fat-dry milk in PBS, pH 7.5, and incubated over night at 4 °C with anti-Bax antibodies. Blots were treated with horseradish-conjugated secondary antibodies for 90 min. Visualization was performed with the enhanced Chemiluminescence's kit from Amersham Pharmacia Biotech. Immunoblot bands were quantified by Scion Image program.

3.8. Statistical Analysis

Data represent the median and range of three separated samples for enzyme activities and lipids and the means ± S.D. of four separate experiments for Bax analysis.

4. Conclusions

Proton beams induced epithelial thyroid cells in a proapoptotic state if the cells were quiescent whereas in an initial apoptotic state if the cells were proliferating, by changing particularly the nuclear SM metabolism. In cell N the strong activation of SMase reduced SM content that was important for the DNA stability. The produced ceramide probably was translocated to the cytoplasm where could be metabolized to sphingosine and sphingosine-1-phosphate, mediators involved in apoptosis. Our data indicate that nuclear SM metabolism is involved in proton-induced damage.

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Author Contributions

Elisabetta Albi designed the experiments, analyzed the data and wrote the paper; Giuseppina Perrella did the experiments and analyzed the data; Andrea Lazzarini, Samuela Cataldi, Remo Lazzarini and Alessandro Floridi did the experiments; Francesco Saverio Ambesi-Impiombato designed the experiments and revised the paper Francesco Curcio designed the experiments and analysed the data.

Conflicts of Interest

The authors declare no conflict of interest.

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COMMENTO DELL' VIII° LAVORO

Risultati hanno mostrato che il fascio di protoni induce un aumento dei livelli di Bax, proteina coinvolta nel processo apoptotico nelle cellule quiescenti e l'effetto era molto più intenso in quelle proliferanti. Nei NFL I protoni aumentavano l'attività della SM sia in cellule proliferanti che in quelle quiescenti con valori simili, mentre a livello nucleare l'aumento dell'attività dell'enzima nelle cellule proliferanti era 12 volte più alto rispetto a quelle quiescenti. L'attività della Smsintasi cambiava soltanto nei nuclei purificati delle cellule quiescenti.

Come conseguenza il rapporto Smasi/Smsintasi aumentava nelle cellule quiescenti rispetto al controllo ma appariva molto più elevato nei N delle cellule proliferanti. Tale risultato, accanto a quello del Bax dimostravano che le cellule trattate con fasci di protoni entravano in uno stato proapoptotico.

VI. DISCUSSIONE

I risultati ottenuti dalla ricerca svolta nel corso del Dottorato hanno chiaramente dimostrato, per la prima volta, gli effetti dell'esposizione a lungo termine ad un ambiente di reale microgravità sulla struttura e funzione della ghiandola tiroidea *in vivo*.

Il disegno sperimentale utilizzato per lo studio è complesso perché: 1) *in vivo* la funzione tiroidea è regolata dall'allse ipotalamo-ipofisario-tiroide; 2) gli animali hanno iniziato il protocollo sperimentale in giovane età e lo hanno terminato da adulti e la funzione tiroidea è correlata all'età (Midgley and Gruner, 1985)

Lo step iniziale della stimolazione della ghiandola tiroidea è l'interazione TSH-TSHr sulla membrana basolaterale dei tireociti inducendo la produzione di cAMP che trasduce il segnale alle protein kinasi A citosoliche (PKAs) (Vassart and Dumont, 1992; Calebiro *et al*., 2006). I nostri risultati hanno mostrato che i follicoli tiroidei degli animali che hanno partecipato alla missione spaziale hanno una struttura più omogenea e organizzata, con una prevalenza di follicoli ordinati e larghi dove i tireociti appaiono più alti e di conseguenza aumenta il rapporto volumetrico tireocita/colloide. Le dimensioni e il volume del follicolo tiroideo è di particolare importanza poiché le modificazioni di tali parametri cambiano il rilascio di cAMP e le conseguenti proprietà dei segnali a cascata attivati (Neves *et al*., 2008; Kholodenko and Kolch, 2008).

Inoltre nei lobi tiroidei degli animali dello spazio il TSHr risulta overespresso. E' noto che il TSHr è un recettore associato alla proteina G che si localizza sia in frazioni della membrana cellulare libera da raft e sia all'interno dei raft ricchi di caveolina 1 dove avviene l'interazione TSH-TSHr; dopo l'esposizione a TSH, il complesso raft-TSHr scompare poiché il TSHr viene scisso in monomeri che

abbandonano il raft (Latif et al., 2003: Latif et al., 2007). I nostri risultati mettono in evidenza che nello spazio si ha una intensa co-localizzazione TSHr-caveolina 1, suggerendo che la microgravità favorisce l'interazione TSHr-raft di membrana. E' noto che i raft di membrana sono ricchi di CHO, SM e gli enzimi per il suo metabolismo quali SMasi e SM-sintasi che regolano l'integrità o meno dei raft stessi (Cascianelli et al., 2008). Nell'ambiente spaziale la SMasi e la SM-sintasi appaiono overespresse; in particolare la SMasi trasloca dal nucleo alla superficie di membrana dove interagisce con il TSHr. E' possibile che la migrazione della SMasi nel raft di membrana dove risiede il TSHr degradi la SM liberando ceramide e rappresentando di conseguenza un momento critico per l'inizio della trasduzione del segnale dopo trattamento con TSH. Infatti, il trattamento con 10^{-7} M TSH stimola il rilascio di cAMP con valori più alti di quelli ottenuti negli animali controllo. Quindi supponiamo dunque che l'assenza di gravità sia un fattore importante che permette un movimento molecolare intracellulare che induce un rimodellamento delle membrane. D'altro canto i nostri risultati dimostrano anche che in assenza di gravità la galectina-3, marker tumorale tiroideo, non solo è overespressa ma abbandona i tireociti per riversarsi nella colloide. Dunque la gravità dovrebbe essere una forza che assicura il corretto posizionamento molecolare per la risposta struttura/funzione tiroidea caratteristica dell'ambiente terrestre. Non è possible escludere inoltre che parte degli effetti riscontrati nella ghiandola tiroidea al rientro dalle missioni spaziali possa essere in parte dovuta a radiazioni poichè è noto che i tireociti sono particolarmente sensibili alle radiazioni (Albi et al., 2008). Infatti, in uno studio parallelo, abbiamo dimostrato che i fasci di protoni inducono i tireociti in uno stato proapoptotico se le cellule sono quiescenti e in un iniziale stato apoptotico se le cellule sono proliferanti, via SM nucleare.

In ogni caso, nell'ambiente spaziale, tutto questo cambiamento molecolare induce una iperfunzione dei follicoli.

Questo però accade a scapito dello spazio parafollicolare e delle cellule C in esso contenute. Infatti, i nostri risultati mostrano una perdita delle cellule parafollicolari e una riduzione di produzione di calcitonina, di notevole importanza per il suo coinvolgimento nel metabolismo osseo.

La perdita della massa ossea è la più importante complicazione per gli astronauti durante le missioni spaziali. Si ritiene che questa sia dovuta ad una eccezionale forma di disuso insieme ad una alterazione del metabolismo dei minerali (Klein-Nulend *et al*., 2003). Accanto a ciò, in dipendenza dalla lunghezza della missione, stress, nutrizione, movimento dei liquidi corporei e deidratazione, gli astronauti vanno incontro ad un quadro osteoporotico caratterizzato da una fragilità ossea con rischi di fratture (Heer et al., 1999). Non vi è alcun dato sulla produzione di calcitonina, un inibitore del riassorbimento osseo osteoclastico, in ambiente spaziale pur essendo noto che il trattamento con calcitonina basale ha una doppia azione sull'osteoporosi e sulla osteoartrite con significativo miglioramento della qualità della vita (Esenyel et al., 2012). I nostri risultati suggeriscono che la riduzione della calcitonina nell'ambiente spaziale possa essere coinvolta nel danno osteoporotico degli astronauti.

Dunque l'ambiente spaziale sembra indurre *in vivo* un'iperfunzionalità delle cellule follicolari alle spese di quelle parafollicolari. Sulla base delle nostre osservazioni è possibile che la reale microgravità induce modificazioni strutturali/funzionali della ghiandola tiroidea con significativi effetti sull'omeostasi endocrina degli astronauti. Ciò è rilevante considerando che gli ormoni tiroidei controllano il metabolismo e la funzionalità di diversi organi ed apparati come il sistema nervoso, cardiovascolare, immunitario, osseo-cartilagineo ecc.

E' possibile ipotizzare due differenti possibilità che potrebbero giustificare i cambiamenti morfo-funzionali della ghiandola tiroidea nello spazio: 1) a causa della lunga durata dell'esperimento, gli animali sono stati analizzati quando sono già diventati adulti con una attività tiroidea più bassa

rispetto all'età giovanile (Elmlinger et al., 2001) ed è possibile che l'ambiente spaziale ritardi l'invecchiamento e mantenga l'attività tiroidea più alta rispetto ai controlli; 2) i cambiamenti tiroidei rappresentano una risposta allo stress. Le condizioni di stress sono generalmente associate ad una diminuizione dei livelli di T3, T4 rispetto alle condizioni controllo (Helmreich et al., 2005). E' possibile che l'aumento di TSHr in ambiente spaziale sia un meccanismo compensatorio allo stress che permette una intensa risposta al TSH. Quest'ultima ipotesi potrebbe spiegare il perché gli animali mantenuti in ambiente confinato a terra (in MDS) presentino condizioni morfo-funzionali della ghiandola tiroidea intermedie tra quelle degli animali nello spazio e quelle di animali mantenuti in laboratorio in normali gabbie. E' possibile che l'ambiente confinato determini una condizione di stress con coinvolgimento della ghiandola tiroidea. Di conseguenza le modificazioni al rientro da una missione spaziale sono la somma degli effetti dell'ipogravità e dell'ambiente confinato.

Al fine di meglio comprendere l'effetto della componente delle fisiche sulle modificazioni molecolari delle cellule tiroidee, i risultati ottenuti in condizioni di ipogravità sono stati confrontati con quelli ottenuti con esperimenti di ipergravità. Nell'ipergravità le tiroidi di topo mostrano le stesse modificazioni di Bax, nonché simili variazioni strutturali e simili variazioni numeriche dei tireociti e delle cellule parafollicolari riscontrate in ipogravità ma la produzione di calcitonina risulta superiore. Inoltre, anche se in ipergravità non si riscontra l'overespressione della SMasi e SM-sintasi come accade in ipogravità, la SMasi trasloca dal nucleo al citoplasma esattamente come in condizioni di ipogravità e ha valori di attività veramente simili. Questo suggerisce che qualsiasi cambiamento della forza di gravità possa essere responsabile di un rimodellamento molecolare che potrebbe influenzare il destino cellulare.

Per studiare se le forze fisiche di gravità determinassero le stesso effetto in un topo normale e in un topo in cui vi sia una overespressione di una proteina utile per la struttura ossea, sono stati utilizzati,

nelle stesse condizioni sperimentali, topi transgenici (TG) overesprimenti pleiotropina, un fattore di stimolazione degli osteoblasti. Le ghiandole tiroidee isolate da topi TG controllo a terra presentano aspetti atipici come una marcata riduzione della superficie follicolare e la comparsa di larghe aree chiare destrutturate accanto a una riduzione di espressione di TSHr, SMasi e SM-sintasi rispetto ai topi WT. Tali dati potrebbero essere dovuti al fatto che il fattore di stimolazione degli osteoblasti è una proteina associata alla matrice extracellulare e che quindi potrebbe essere presente anche nella matrice della tiroide; di conseguenza una sua overespressione potrebbe disorganizzare la struttura tiroidea. In ambiente spaziale le modificazioni riscontrate nei topi WT sono attenuate nei topi TG, suggerendo che l'overespressione della pleiotropina possa esercitare un effetto protettivo con un possibile effetto indiretto. Tavella *et al* (2012) hanno dimostrato che l'ambiente spaziale induce una perdita delle trabecole ossee più evidente nei topi WT rispetto ai TG, indicando che l'overespressione della pleiotropina previene il danno osseo indotto dalla ipogravità. E' possibile che il più alto contenuto di calcitonina nell'ambiente spaziale in topi TG rispetto a quelli WT possa partecipare all'effetto protettivo della overespressione di pleiotropina la quale esercita lo stesso effetto anche rispetto ai danni tiroidei indotti da ipergravità.

VI. Conclusioni

Nell'ambiente spaziale la ghiandola tiroidea subisce delle modificazioni sia nella sua componente follicolare e sia in quella parafollicolare partecipando in modo significativo ai danni riscontrati degli astronauti al rientro dalle missioni spaziali. La comprensione dei meccanismi che sono alla base di tali cambiamenti sono utili per la possibilità di adottare contromisure mirate al mantenimento della salute degli astronauti.

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