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Sarcoglycans and integrins in human thyrocytes: an immunofluorescence study

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Abstract

The sarcoglycan sub-complex is a protein system which plays a key role in sarcolemma stabilization during muscle activity consisting of six glycosylated transmembrane proteins. Integrins play a key role in the process of cell adhesion, linking the extracellular matrix to the actin cytoskeleton. Here we have analysed the receptor for thyroid hormone, identified on αvβ3-integrin that has an important role in the activation of non-genomic actions of the hormone. Many non-genomic actions of the thyroid hormone appear to contribute to basal levels of activity of a variety of proteins, including ion pumps, intracellular protein trafficking and protein turnover. The purpose of our research was to study the presence and behaviour of sarcoglycans and integrins on the thyroid gland, in both normal and pathological conditions, for the first time. Our results show a normal fluorescence pattern in patients without pathology, and a reduced fluorescence pattern in patients with thyroid disease. Moreover, colocalization in healthy patients was found in double localization reactions, whereas in patients with Hashimoto's thyroiditis sarcoglycans did not colocalize with tested integrin. These data could confirm the hypothesis of a close association between sarcoglycans and integrins, which, in pathological condition, are not found contemporarily hypothesizing that each single protein system could have a role in maintaining cell vitality.

Keywords

sarcoglycans; thyrocytes; integrin; immunofluorescence; thyroid.

Introduction

The sarcoglycan sub-complex (SGC) is a multi-member transmembrane complex which provides a connection between extracellular matrix components and the cytoskeleton. The SGC is made up of six glycoproteins, α-, β-, γ-, δ-, ε- and ζ-sarcoglycan (SG), linked by lateral binding to β- dystroglycan. β-, γ-, δ-, and ζ-SGs are similar to type II transmembrane proteins with the NH-terminal on the intracellular side; α- and e-SG is a type I transmembrane proteins with the NH-terminal on the extracellular side (1-3). Furthermore, ζ-SG, similar to δ- and γ-SGs has been identified and has also been found as a component of the vascular smooth muscle SGC (4).

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In recent years, SGs have been the most studied proteins, together with dystrophin, which plays an important role in the pathogenesis of many muscular dystrophies (e.g. Duchenne and Becker muscular dystrophies). Molecular genetic studies have allowed to demonstrate that a mutation in each single SG gene causes a series of recessive autosomal dystrophin-positive muscular dystrophies, known as sarcoglycanopathies or limb girdle muscular dystrophies (LGMD type 2D, 2E, 2C and 2F) (5-9).

Our previous studies on skeletal muscle fibers have demonstrated that SGs are organized in costameres representing a protein machinery made up of a dystrophin-glycoprotein complex and a vinculin-talin-integrin system, proving the colocalization of SGs and integrins in adult human skeletal muscle and supporting the hypothesis of the existence of bidirectional signalling between SGs and integrins (10-12).

Integrins, part of the vinculin-talin-integrin system, are transmembrane heterodimeric receptors, that play an important role in the process of cell adhesion, linking the extracellular matrix to the actin cytoskeleton (13).

These proteins would appear to be the main mediators of cellular processes such as adhesion, migration, survival, apoptosis, and cell differentiation (14) and they are primarily expressed in skeletal muscle fibers and in cardiac and smooth muscle fibers (15, 16).

Our previous studies, carried out on human biopsies obtained from airway, gastrointestinal, and urinary tracts, demonstrated the presence of all SGs in smooth muscle tissue (17, 18). In particular, all six sarcoglycans are organized in different tetramers, or even in a hexameric arrangement (18) Nevertheless, it is not clear yet what are the tetramers in these tissues. Also in glandular epithelium from the mammary and prostatic gland it was possible to demonstrate a normal presence of all SGs in healthy tissues and a reduced fluorescence in samples with mammary or prostatic pathology (19, 20). In particular, the study on mammary gland showed that in biopsies of normal breast tissue, immunofluorescence was detectable for all the tested SGs, the staining pattern for all SGs was distributed in all cells and immunofluorescence for all SGs was detectable also in myoepithelial cells. In samples of pathological breast tissue, we observed that staining for all the tested SGs appeared to be severely reduced or absent, both in epithelial and myoepithelial cells (19); these studies have demonstrated that the lack of SGs, observed in the biopsies of breast tissue obtained from patients with fibroadenoma and fibrocystic mastopathy, may provoke a loss of strong adhesion between epithelial cells, facilitating degeneration and progression from benign tumours in malignant tumours.

Similar results we have obtained in glandular epithelium of prostatic gland, analysing samples of patients affected by benign prostatic hyperplasia and by prostatic adenocarcinoma (20).

Based on our previous results in epithelial and glandular tissues, in the present report we performed a study on SGs and integrins in thyrocytes. In particular, we studied all SGs and the $\alpha v\beta 3$- integrin, a cell surface receptor for thyroid hormone, which is the initiation site for T4-induced activation of intracellular signalling cascades (21) by activation of the sodium pump (14, 22). As this integrin is also widely present on cancer cells, it was clear that the thyroid hormone has also been found to cause proliferation of a variety of human cancer lines via the cell surface receptor. The thyroid hormone analogue L-thyroxine (T4), widely studied in genomic mechanisms of hormone action as a prohormone antecedent to T3 through tissue deiodinas-

es, is biologically active at the integrin receptor (23). This integrin is considered the main exponent of that initiation/progression process that leads to many human diseases, both inflammatory and neoplastic (24-26).

Since the presence of SGs and their interactions with integrin, in thyroid gland epithelium, has not previously described, here we have analysed the behaviour of these transmembrane proteins, in healthy and pathological subjects, by immunofluorescence techniques. Relatively to pathological conditions, we have analysed samples of thyroid obtained by patients affected by Hashimoto's thyroiditis, since it is considered a risk factor for thyroid cancer (27).

Materials and methods

Patients

For the data obtained at the level of the thyroid gland, thyroid needle aspiration was used for the this study, a minimally invasive method that allows, in most cases, a precise diagnosis on the nature of thyroid nodules. Thyroid needle aspiration was performed according to the American Association of Clinical Endocrinologists (AACE), American College of Endocrinology (ACE) and Associazione Medici Endocrinologi (AME) 2016 guidelines. Cytological sampling was performed using a thin needle, 20-24 G, attached to a syringe by suction technique. Given the minimum duration and minimally invasive blood sampling, the 12 patients were not subjected to local anaesthesia. The needle aspiration was also performed under continuous ultrasound guidance. The ultrasound guide allowed us to pick up cells also from non-palpable nodules, to choose which nodule or part of the nodule to subject to needle aspiration and to increase the diagnostic power of the needle aspirator (28, 29). In detail, needle aspiration was performed on 12 patients: 2 control patients (female), affected by colloidal cysts, and 10 patients with Hashimoto's thyroiditis (8 female and 2 male), aged between 26 and 77 years. Once the sampling was carried out, we performed the preparation of the material by direct smear on slide. All patients gave their informed consent for the treatment of the cytology obtained by needle aspiration, for scientific purposes.

Immunofluorescence

The biopsies were fixed in 3% paraformaldehyde in 0.2 M phosphate buffer, ph. 7.4, for 2 hours at room temperature. They were washed extensively with 0.2 M phosphate buffer ph. 7.4, and then with phosphate buffer saline (PBS), containing 12 and 18% sucrose. The samples were snap-frozen in liquid nitrogen, and 20 μm sections were prepared in a cryostat for use in a protocol to perform immunofluorescence. The sections were placed on glass slides that were coated with 0.5% gelatine and 0.005% chromium potassium sulphate.

To block nonspecific binding sites and to permeabilize the membranes, the sections were pre-incubated with 1% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS for 15 minutes at room temperature. Finally, the sections were incubated with primary antibodies. The following primary polyclonal anti-goat antibodies were used: anti-

α-SG diluted 1:100, anti-β-SG diluted 1:200, anti-γ- SG diluted 1:100, anti-δ-SG diluted 1:10, anti-ε-SG diluted 1: 100, anti-ζ-SG diluted 1: 100, anti-αvβ3-integrins diluted 1:100 (all from Santa Cruz Biotechnology Inc., Santa Cruz, Ca., USA). About SGs, primary antibodies were detected using Texas Red-conjugated IgG anti-goat diluted at 1:250 (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa., USA), about αvβ3-integrin, primary antibody was detected using fluorescein isothiocyanate (FITC) IgG-conjugated anti-mouse, (1:250 dilution; Sigma Chemicals, St. Louis, Mo., USA)

The sections were then analysed and images acquired using a Zeiss LSM 5 DUO (Carl Zeiss, Jena, Germany) confocal laser scanning microscope. All images were digitized at a resolution of 8 bits into an array of 2048 x 2048 pixels. Optical sections of fluorescent specimens were obtained using a HeNe laser (wave-length 543 nm) and an Ar laser (wavelength 458 nm) at a 62 second scanning speed with up to 8 averages; 1.50μm thick sections of fluorescent specimens were obtained using a pinhole of 250. Contrast and brightness were established by examining the most brightly labelled pixels and choosing the settings that allowed clear visualization of the structural details while keeping the pixel intensity at its highest (~200). Each image was acquired within 62s, in order to minimize photodegradation.

For image analysis, we used splitting, and the function called Histo, both belonging to software ZEN 2009 of the ZEISS LSM DUO. In particular, the splitting shows individual channels and relative mergers; the Histo allows to display a histogram of entire image calculating the distribution of pixel intensities and showing the fluorescent values in a table form wherein the mean intensity, standard deviation and number of pixel are indicated. The mean intensity and standard deviation for each images were reported in Tab. 1. Number of pixels was always the same because the images were all the same size and therefore not shown in the table.

Digital images were cropped and figure montages were prepared using Adope Photoshop 7.0 (Adobe system, Palo Alto, Calif. , USA).

Results

In this study, we performed immunofluorescence analysis of the thyroid tissue samples using antibodies against all SGs and against αvβ3-integrin. The 20-μm-thick cryosections were analysed by acquiring with a scan step size of 0.8μm.

Immunofluorescence investigations were performed in single-localization reactions analysing singularly SGs and αvβ3-integrin, and in double-localization reactions combining single SGs with αvβ3-integrin. The immunofluorescence reactions were carried out both in control thyrocytes and in thyrocytes during pathological conditions.

In particular, single-localization reactions (Fig. 1) using antibodies against α-SG (A), β-SG (B), γ-SG (C), δ-SG (D), ε-SG (E), and ζ-SG (F) in thyrocytes obtained from healthy subject samples show a normal fluorescence pattern for all tested proteins.

We observed the same result using antibodies against αvβ3-integrin in thyrocytes taken from healthy subject samples. Also in this case the fluorescence pattern for tested protein shows a normal staining (Fig. 2).

Instead, performing a single-localization reaction with antibodies against α-SG (Fig. 3A), β-SG (Fig. 3B), γ-SG (Fig. 3C), δ-SG (Fig. 3D), ε-SG (Fig. 3E), and ζ-SG (Fig.

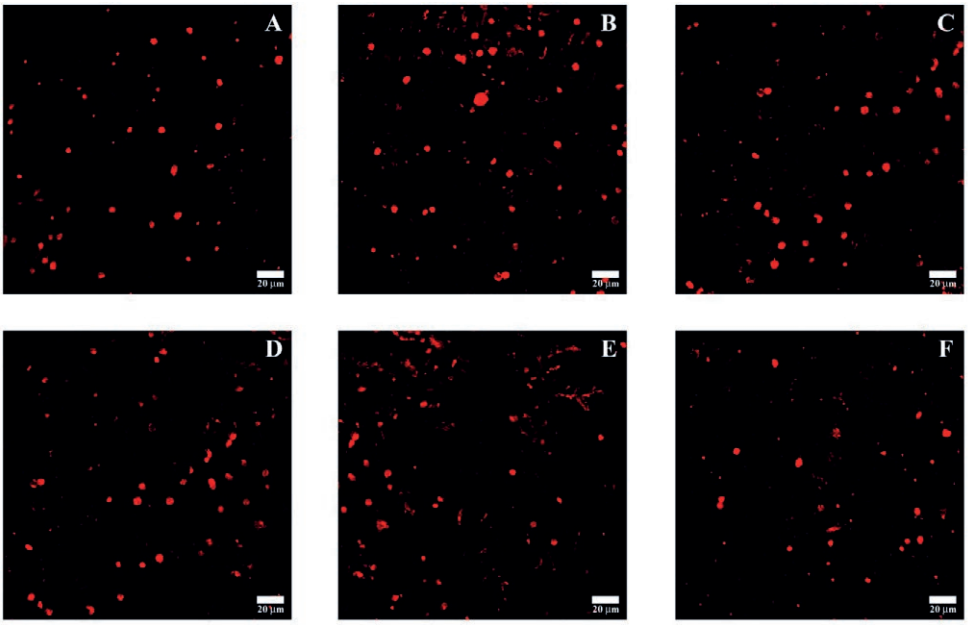

Figure 1. Compound panel showing immunofluorescence findings in thyrocytes obtained from healthy subjects. The samples were immunolabelled with single-localization reaction using antibodies against α-sarcoglycan (A), β-sarcoglycan (B), δ-sarcoglycan (C), γ-sarcoglycan (D), ε-sarcoglycan (E), ζ-sarcoglycan (F). All tested proteins show a normal staining pattern.

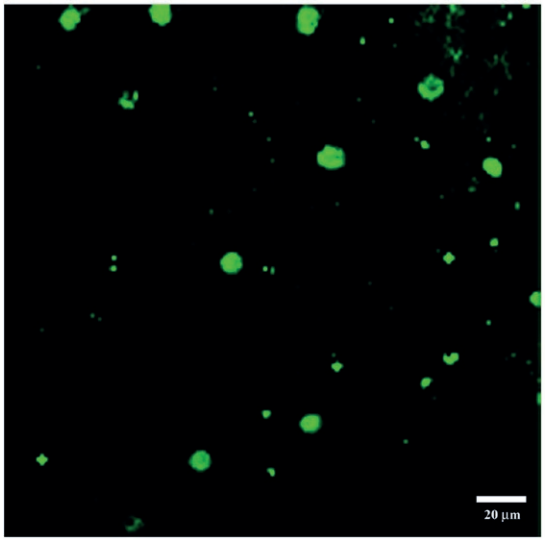

Figure 2. Single-localization reaction performed using antibodies against integrin αvβ3 showing a normal staining pattern for this protein in thyrocytes obtained from healthy subject.

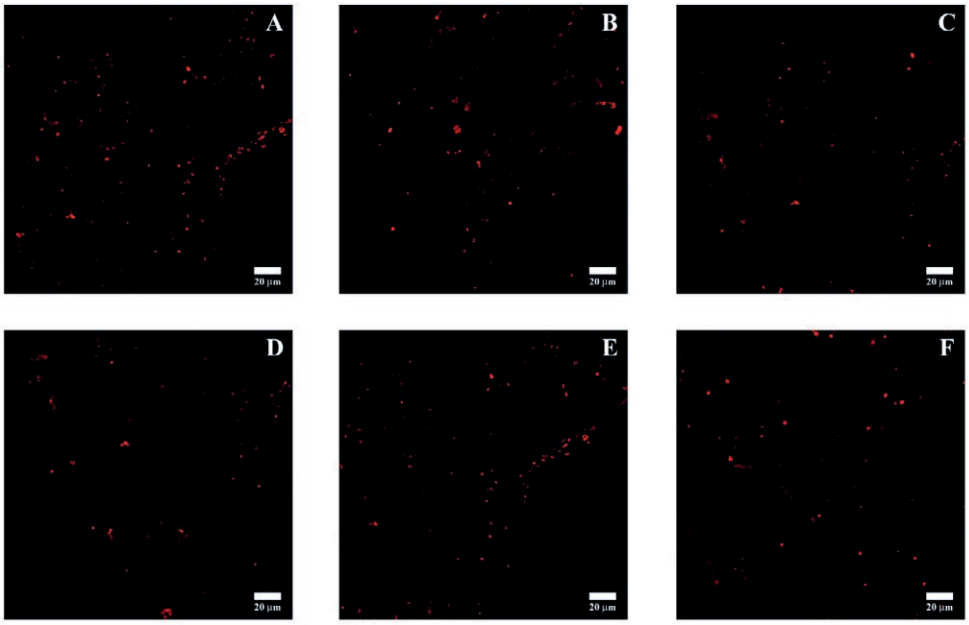

Figure 3. Compound panel showing immunofluorescence findings in thyrocytes obtained from subjects affected by Hashimoto's thyroiditis. The samples were immunolabelled with single-localization reaction using antibodies against α-sarcoglycan (A), β-sarcoglycan (B), δ-sarcoglycan (C), γ- sarcoglycan (D), ε-sarcoglycan (E), ζ-sarcoglycan (F). All tested proteins show a decreased staining pattern.

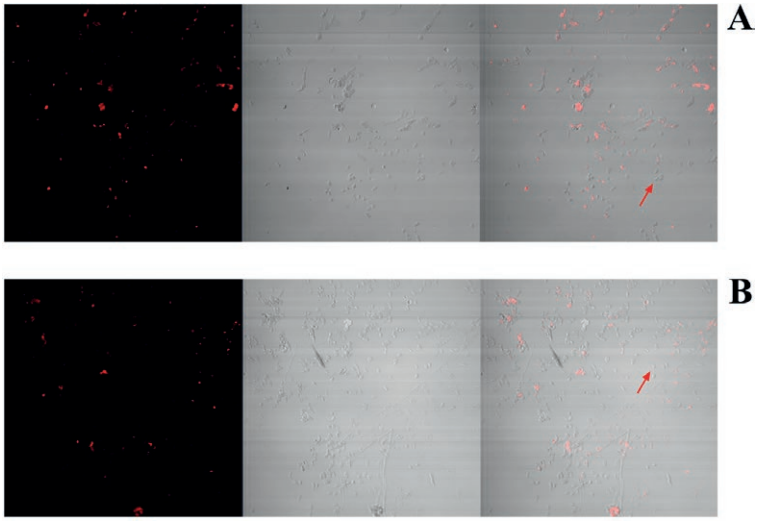

Figure 4. Single-localization reactions performed with antibodies against β- (A) and δ-sarcoglycan (B) respectively showed in Figure 5B and 5D. Merging the fluorescence channel (red channel) with transmitted light (gray channel) in the images to the right, it is possible to denote the absence of staining pattern in some cells. (red arrows).

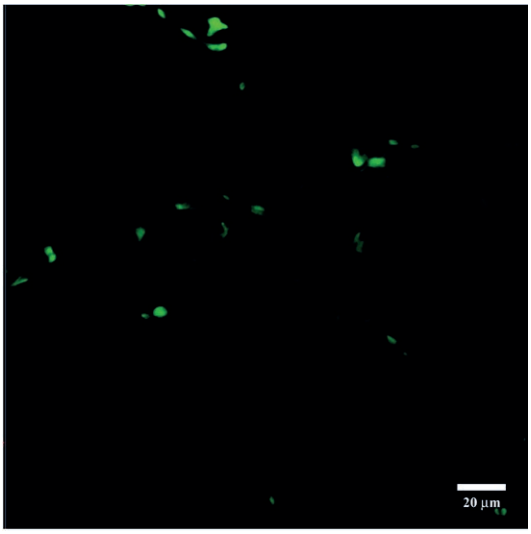

Figure 5. Single-localization reaction performed using antibodies against αvβ3-integrin showing a decreased staining pattern for this protein in thyrocytes obtained from subjects affected by Hashimoto's thyroiditis.

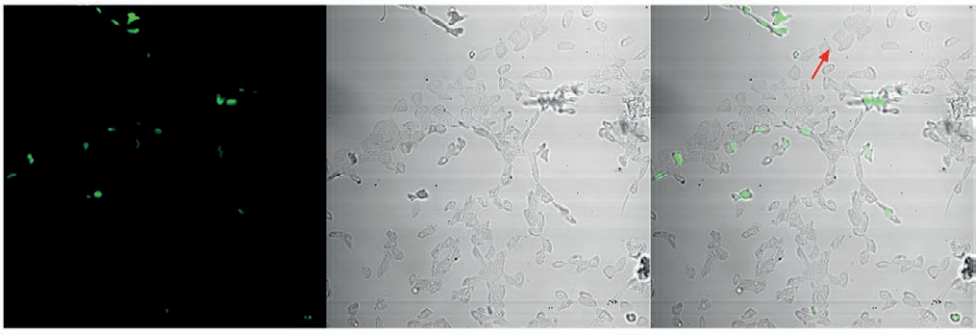

Figure 6. Single-localization reactions performed with antibody against αvβ3-integrin shown in the previous figure. Merging the fluorescence channel (green channel) with transmitted light (gray channel) in the images to the right, it is possible to denote the absence of staining pattern in some cells (red arrow).

3F) in thyrocytes obtained from subjects with Hashimoto's thyroiditis, a decreased pattern of all SGs in these cells can be observed, compared to thyrocytes obtained from healthy subjects.

Merging the fluorescence channel (red channel) with transmitted light (grey channel) using antibodies against β-SG (Fig. 4A) and δ-SG (Fig. 4B), respectively, shown in previous Figure 3B and 3D, it is possible to show the absence of staining pattern for sarcoglycans in some cells (red arrows). The same condition was observed for other SGs (data not shown).

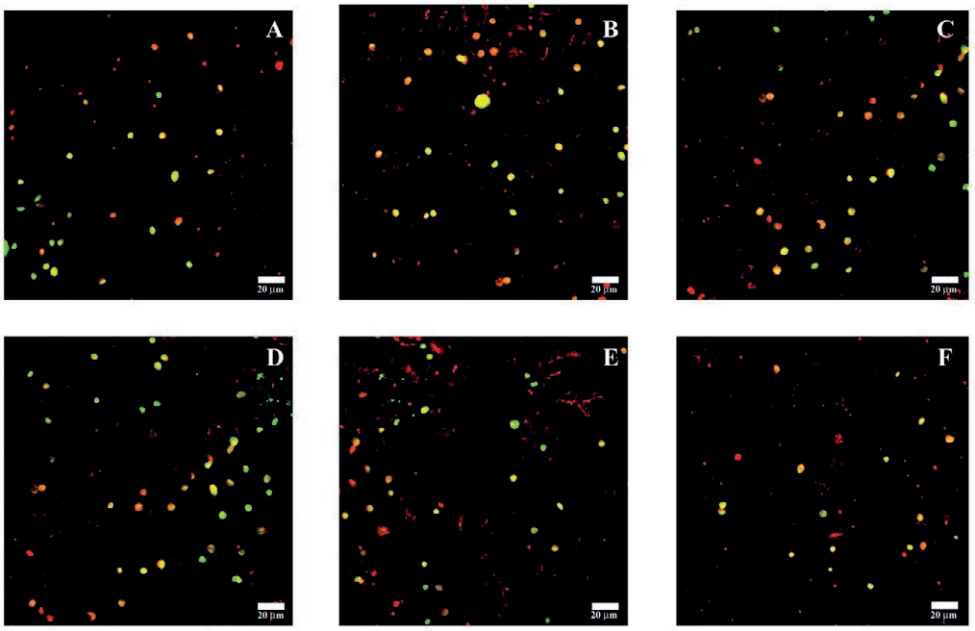

Figure 7. Compound panel showing immunofluorescence findings in thyrocytes obtained from healthy subjects. The samples were immunolabelled with double-localization reaction analysing antibodies against α-sarcoglycan (A), β-sarcoglycan (B), δ-sarcoglycan (C), γ-sarcoglycan (D), ε-sarcoglycan (E), ζ-sarcoglycan (F) (all in red channel) and αvβ3-integrin (green channel). It is possible to denote a yellow fluorescence due to an overlapping of red fluorescence (sarcoglycans) on green fluorescence (integrin) demonstrating a co-localization between these proteins.

Single-localization reaction carried out using antibody against αvβ3-integrin in thyrocytes obtained from subjects affected by Hashimoto's thyroiditis shows a decreased staining pattern for this protein compared to results obtained in thyrocytes taken from healthy subject samples (Fig. 5).

Merging the fluorescence channel (green channel) with transmitted light (grey channel) in single-localization reaction using antibody against αvβ3-integrin (Fig. 6A), it is possible to denote the absence of tested protein in some cells (red arrow), as seen for SGs.

Subsequently, we carried out double-localizations, analysing each single SG with αvβ3-integrin in thyrocytes obtained from healthy subject samples. In particular, superimposing α-SG (Fig. 7A), β- SG (Fig. 7B), γ-SG (Fig. 7C), δ-SG (Fig. 7D), ε-SG (Fig. 7E), and ζ-SG (Fig. 7F), shown with red channel, and αvβ3-integrin, shown with green channel, we observed a yellow fluorescence due to an overlapping of red fluorescence (SGs) on green fluorescence (integrin) demonstrating a co- localization between these proteins.

Double-localization reaction performed combining α-SG (Fig. 8A), β-SG (Fig. 8B), γ-SG (Fig. 8C), δ-SG (Fig. 8D), ε-SG (Fig. 8E), and ζ-SG (Fig. 8F), with αvβ3-integrin in thyrocytes obtained from patients affected by Hashimoto's thyroiditis show the

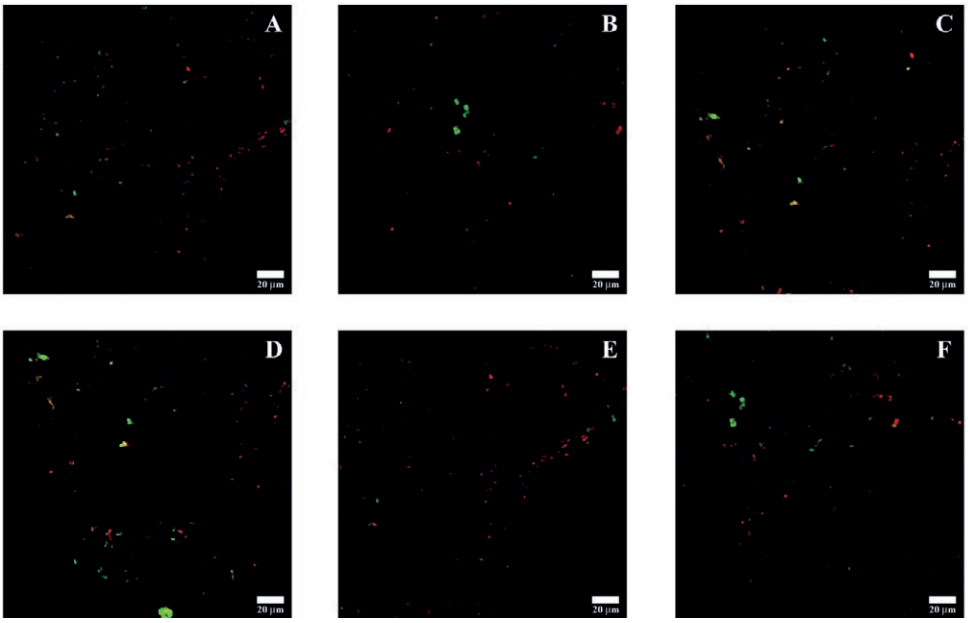

Figure 8. Compound panel showing immunofluorescence findings in thyrocytes obtained from subjects affected by Hashimoto's thyroiditis. The samples were immunolabelled with double-localization reaction analysing antibodies against α-sarcoglycan (A), β-sarcoglycan (B), δ-sarcoglycan (C), γ- sarcoglycan (D), ε-sarcoglycan (E), ζ-sarcoglycan (F) (all in red channel) and αvβ3-integrin (green channel). It is possible to denote the presence of the cells expressing either only red fluorescence or only green fluorescence, but always in a reduced amount compared to thyrocytes obtained from healthy subjects.

Table 1. Histo function applied in each immunofluorescent image in single-localization showing the mean intensity and standard deviation of real pixel intensity in entire image (M.I.= Mean Intensity; S.D.= Standard Deviation).

Protein	Control		Hashimoto's thyroiditis	
	M.I.	S.D.	M.I.	S.D.
α-SG	5.97	28.485	1.94	16.818
β-SG	6.79	35.041	1.48	14.651
γ-SG	5.21	30.267	0.96	11.063
δ-SG	4.98	28.152	1.02	11.146
ε-SG	6.04	31.012	1.91	16.823
ζ-SG	4.87	24.716	1.44	14.745
αvβ3-integrin	5.87	26.823	1.63	19.375

presence of cells expressing either only red fluorescence or only green fluorescence, but always in a reduced amount compared to thyrocytes obtained from healthy sub-

jects, indicating a no-colocalization between SGs and tested integrin.

In order to display the real values of the pixel intensity corresponding to tested proteins, for each previous images in single-localization, the function called Histo was applied and the mean intensity and standard deviation were reported in Tab. 1. In this table, really it was possible to denote how the pathological thyrocytes show low values compared to those of the control thyrocytes.

Discussion

Sarcoglycans are a subcomplex of glycoproteins mediating interaction between the extracellular matrix and sarcolemma of the myofibers, protecting also the muscle fibers from damage provoked by contraction and relaxation (30-33). In our previous studies on SGC, we examined smooth muscular districts, both in normal and pathological conditions, finding that in smooth muscle the SGC presented a hexameric structure (formed by all SGCs) which has a higher or lower expression of a single SG in conformity with the function of smooth muscle in the gastrointestinal, urogenital, vascular or respiratory tract (17, 18, 34).

However, it has been shown that only α- and γ-SG constantly are present in striated and smooth muscle, whereas other SGs are identified also in other tissues (2). Indeed, it has been hypothesized the presence of a bidirectional signaling between SGs and integrin adhesion system in cultured L6 myocytes (10).

Extracellular domains of the integrins, a known family of transmembrane glycoproteins, interact with a variety of ligands (35), including extracellular matrix glycoproteins, and the intracellular domain linked to the cytoskeleton (36). Moreover, it has been demonstrated that the thyroid hormone could influence the interaction of integrins with the extracellular matrix protein (22). At this purpose, $\alpha v\beta 3$-integrin has a large number of extracellular protein ligands, including growth factors and extracellular matrix proteins. In particular, the role of $\alpha v\beta 3$-integrin as a cell surface receptor for thyroid hormone at which non-genomic actions are initiated and as a mediator of thyroid hormone effects on plasma membrane ion transporters and on intracellular protein trafficking has been analyzed (37).

Then, in this work we carried out primarily a semiquantitative study on thyrocytes obtained from unaffected subjects, in order to analyze and to understand the behavior of SGs and $\alpha v\beta 3$-integrin in non-muscle cells.

In the present study, immunofluorescence results demonstrated, for the first time, that in thyrocytes of healthy patients: (i) all tested SGs were detectable with normal staining pattern in all analysed cells; (ii) $\alpha v\beta 3$-integrin was detectable in all analysed thyrocytes; (iii) each SGs co-localize with $\alpha v\beta 3$-integrin.

Our previous results on normal breast and prostatic epithelium have shown a clearly detectable immunofluorescence for all SGs, normally distributed in all cells, demonstrating a hexameric structure for this subcomplex (19, 20). Based on our present results, we can assert that also in thyrocytes exists a SGC structured with hexameric organization. Moreover, the present data, evidencing a constant colocalization between SGs and integrin, confirm that also in thyrocytes exists a bidirectional signalling between these proteins, as previously demonstrated in skeletal and smooth muscle (11, 12, 34) and that also in thyrocytes a protein machinery could be present.

Furthermore, as it has been demonstrated that the mutations in any SG cause limb-girdle muscular dystrophy (38, 39) provoking dysphagia and acute digestive dilatations (40), it was possible to assert that SGs play an important role in ethiopathogenesis of muscular pathologies and they seem to be functionally and pathologically as important as dystrophin (10). Moreover, our previous reports on breast and prostatic epithelium, have demonstrated a clear reduction or absence of SGs in samples obtained from pathological subjects (19, 20). On this basis, in order to understand the real role of SGs and αvβ3-integrin, it was necessary to analyze these proteins also in pathological conditions studying SGs and αvβ3-integrin in thyrocytes obtained from patients affected by Hashimoto's thyroiditis, as well known, risk factor for thyroid cancer (27). Our data on pathological thyrocytes revealed: (i) a severely reduced or almost absent staining pattern for all SGs and αvβ3-integrin; (ii) cells expressing exclusively SGs or exclusively αvβ3- integrins.

These data are similar to our previous results obtained in breast and prostatic epithelium in pathological conditions (19, 20) showing, for the first time, that SGs in normal epithelial cells display a wider distribution compared to pathological tissues in which all SGs are reduced; then, it can possible to hypothesize that SGs and integrins play a key role in regulation of vital functions of the cells also in thyrocytes. Moreover, on the basis of our present data showing the presence of the cells expressing exclusively SGs or exclusively αvβ3-integrins, we can also assert that in pathological conditions the structural and functional roles of these proteins can be reduced. In our opinion, it's possible to hypothesize that a self-defence system is activated to maintain cellular vitality. This system could be activated by SGs when there are no αvβ3-integrins and could be activated by integrins when there are no SGs.

Furthermore, while in normal conditions in the presence of both SGs and αvβ3-integrins, the opening of the membrane ion pumps and the leakage of T3 and T4 from the cells with normal thyrocyte function exists (14), in pathological conditions the reduction of thyrocytic functions could provoke a lack of opening of membrane ion pumps. This confirms that the tested proteins in the present study can play a key role in mediating signalling between the intracellular environment and the extracellular matrix, as evidenced by the reduction of these proteins in pathological conditions, and by the constant colocation between SGs and the αvβ3-integrin. This hypothesis could be confirmed by evidence that the inoculation of integrins in rats affected by autoimmune pathology reduces or abolish infiltration of lymphocytes (41) and so this finding could lead to future therapies.

Our present data, confirming the importance of SGs and αvβ3-integrin also in nonmuscle cells and showing a possible role of these proteins in pathogenesis of thyroiditis, reveal a new avenue of research. In this way, it will possible to understand the variations of SGC in many other tissues and their function in other pathological conditions also integrating these studies with molecular biology techniques.

Authors' contribution

AFa, GC and GR analyzed the data, and wrote the manuscript; GR, AFu and AC designed the experiments, performed the experiments; AC and AF conceived the study and revised the manuscript; GS and SP provided the patient samples.

Ethics approval and consent to participate

The study was performed in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of Istituto Dermopatico dell'Immacolata IRCCS (IDI-IRCSS) of Rome (Sezione Comitato Etico IRCCS Lazio (no. 523-1). The participants provided written informed consent to participate in this study.

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