Clinical and Experimental Health Sciences

Effects of SPARC and Possible Receptors on Colon Cancer Cell Line

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Received: 01.02.2022 **Accepted:** 15.04.2022

ABSTRACT

Objective: The aim of this study was to observe the apoptotic/cytotoxic effects of exogenous SPARC on colon cancer cell line HT-29, then to investigate the function of stabilin-1 and integrin αvβ3, which are possible receptors for SPARC in colon cancer cells and to determine the quantitation of their receptor numbers.

Methods: Appropriate doses of exogenous SPARC and it's inhibitor, cilengitide added to HT-29 cell line were determined by xCELLigence Real-Time Cell Analysis system, SPARC-mediated caspase 3 expressions were measured. Using the RT-PCR system, gene expression levels of SPARC, stabilin-1 and integrin αvβ3 receptors (silenced/nonsilenced with cilengitide) were detected then the numbers of receptors per cell were quantitated by flow cytometry.

Results: IC50 value of SPARC was determined as 4.57 µg/mL and IC50 value of cilengitide was determined as 50 nM. 5 µg/mL exogenous SPARC caused increased apoptosis in the HT-29 line. Significant increase in gene expression of integrin αvβ3 receptor was observed in the group incubated with 5 µg/mL SPARC, contrarily, the addition of cilengitide decreased gene expressions. The integrin αvβ3 receptor numbers increased approximately 2-fold with SPARC compared to the control. No significant changes were observed in the gene expression and receptor numbers of stabilin-1.

Conclusion: Exogenous SPARC was shown to reduce proliferation and induce apoptosis in colon cancer cells. Integrin αvβ3 is thought to be the possible receptor mediating SPARC in colon cancer cells. Quantification of surface receptors per cell, which we think we have done first, can be considered as a marker in the follow-up of anticancer treatments.

Keywords: SPARC, cilengitide, colon cancer, stabilin-1, integrin αvβ3

1. INTRODUCTION

SPARC is a human protein that is encoded by the SPARC gene. It is also known as osteonectin or basement membrane protein 40 (BM-40). Osteonectin is a 40 kDa, acidic, cysteine-rich glycoprotein, which has a single polypeptide chain consisting of three structural domains. The first domain is the calcium binding region (low affinity) located at the N-terminal end and consists of 52 amino acids. The second domain is the cysteine-rich (containing 10 cysteine residues) FS-like (FS: Follostatin) region and consists of 85 amino acids (Asn53-Pro137). The third domain is the EC-binding (EC: extracellular calcium) site. The last domain, the C-terminal end, consists of 149 amino acids (Cys138-Ile286) and has high calcium binding affinity. This region inhibits cell division, proliferation, and adhesion while matrix methyloproteinases induce communication between the cell and the matrix (1-3).

SPARC is involved in wound healing, cell migration and angiogenesis by regulating interactions and communications between cells. While it may also exert anti-angiogenic activity; the role of osteonectin in colorectal cancer is controversial (4). Colorectal cancer is the third most prevalent diagnosed cancer in men and the second in women known worldwide (5) and is a group of diseases with complex genetic and epigenetic features (4). A study, investigating the relationship between the hypermethylation of CpG islands and SPARC in colon cancer tissues, indicated that SPARC expression was downregulated in colorectal cancer due to abnormal methylation of CpG islands in the promoter region (6). It has

DOI: 10.33808/clinexphealthsci.1100770

been concluded that methylation of CpG islands in colon cancer is suppressed in the presence of SPARC (6).

In advanced cancers, a large number of stabilin-1 positive macrophages associated with poor prognosis have been observed, revealing the effect and importance of the overexpression of this receptor on macrophages in carcinogenesis (7).

Cell surface receptors, integrins, are the most important family of cell adhesion receptors which regulate the interaction between cells and extracellular matrix (ECM) proteins. The integrin family consists of at least 24 sub-subunits; 18α and 8β subunits connected by non-covalent bonds (8).

Originally called the vitronectin receptor, αvβ3 integrin is one of the most complex receptors that binds at least 21 different extracellular matrix proteins, including osteopontin, laminin, fibrinogen, thrombospondin, thrombin, and von Willebrand factor (9,10). With its non-covalent heterodimer structure consisting of 170 kDa αV / CD51 and 93 kDa β3 / CD61 subgroups, this integrin is widely expressed in smooth muscle cells (SMC), myofibroblasts, osteoclasts, endothelial cells (EC), monocytes, and platelets (9, 11).

As integrins are the main regulators of communication between cells and with the microenvironment, they play important roles in various cellular processes such as cell migration, differentiation, and survival (12, 13). Therefore, integrins have been targeted for cancer therapy in recent years, and RGD-containing peptides have been developed and synthesized to directly inhibit this receptor (14, 15). The cilengitide molecule, the cyclic Arg-Gly-Glu (RGD) pentapeptide, is an integrin inhibitor. There are several different integrin subtypes that recognize and bind to RGD. Cilengitide has been shown to act as an inhibitor in angiogenesis and induces apoptosis through inhibition of the interaction between integrins and ECM ligands (16, 17). Cilengitide has been found to affect the adhesion between integrin αvβ3 and its ligands and induce apoptosis in cells expressing integrin αvβ3 and αvβ5 with *in-vitro* studies (16).

Herein, we aimed to determine the apoptotic and cytotoxic effects of exogenous SPARC protein on colon cancer cell line HT-29 cells. Furthermore, we investigated the functions of stabilin-1 and integrin αvβ3 (by inhibition with the relevant peptide), possible receptors mediating effects of SPARC in colon cancer. These two receptors selected among many receptors will help us explain the mechanism of action of SPARC protein in colon cancer cells, and the measurement of the number of receptors per cell will guide other studies as a new marker that can be used in cancer treatment.

2. METHODS

2.1. Cell Line

The colon cancer cell line, HT-29 was used in this study. Cells were cultured in DMEM medium containing high glucose supplemented with 10% heat inactivated fetal bovine serum and 100 IU/mL penicillin/streptomycin solution. The cells were cultured at 37°C in a tissue culture incubator containing 5% CO_2 under humid environment, passaging once cells were reached 80% confluency by trypsin-EDTA solution.

2.2. Cell Lysis and Total Protein Determination

For protein isolation, cells were washed with cold phosphate buffered saline solution (PBS) followed by the addition of the lysis buffer and the cells were scraped off. Homogenization was achieved by sonicating cells for 2 minutes with an ultrasonic homogenizer. Proteins were separated from cell membrane residues by centrifugation at +4°C and, 14000 rpm for 10 minutes. Protein concentration was measured by the Bradford method (18).

2.3. Cytotoxicity Test

Cytotoxicity was determined by xCELLigence real-time cell analysis system (xCELLigence, Roche, CA, USA), which measures the changes in electrical impedance created by cellular adhesion to the cell cultureware in which the cells are seeded (19). As the cells divide, the micro electrodes record the the impedance alterations. The system records the electrical changes simultaneously, so that quantitative values about the proliferation of cells are obtained. For evaluating cytotoxicity, $1x10^4$ cells were seeded as triplicates and incubated for 24 hours to allow adherence. Fresh culture media containing SPARC protein at concentrations of 2.5 µg/mL, 1 µg/mL, 0.5 µg/mL and 0.1 µg/mL was added in wells, and its effect on cellular viability was examined for 72 hours, followed by calculation of the IC_{50} value. Cytotoxicity of cilengitide was also evaluated in a similar manner; cells were treated with cilengitide at concentrations of 250 nM, 125 nM, 50 nM, 25 nM and 5 nM for 48 hours and the IC_{50} value was calculated.

2.4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The isolation kit (Cat no: 12183025, Ambion/by Life Technologies, MA, USA) was used to isolate mRNA. Using Nanodrop technology (Epoch, Biotech Ins., CA, USA), the quantity and purity of RNA were measured spectrophotometrically. The High Capacity cDNA Reverse Transcription Kit (Cat no: 4374966, ThermoFisher, MA, USA) was used to reverse transcribe RNA according to the manufacturer's procedure. Real-time polymerase chain reaction (qPCR, Biorad, CA, USA) was used to determine gene expression levels using TaqMan Gene Expression Assays (Invitrogen/Life Technologies, Carlsbad, CA, USA). Hs00234160_m1 (SPARC), Hs01109068_m1 (STAB), Hs01547673_m1 (integrin subunit alpha 5), Hs01105177_m1 (integrin subunit beta 3), and Hs0275899_g1 (GAPDH), all of which are human genes encoding osteonectin, stabilin 1, integrin subunit alpha 5, and integrin subunit beta 3 binding protein. As a housekeeping gene, GAPDH was employed.

2.5. Caspase-3 Activity

Caspase-3 activity was determined by Millipore Colorometric Activity Test Kit (Cat no: APT 165, Sigma-Aldrich, MA, USA) according to the manufacturer's instructions. Total protein samples were collected and their concentrations were measured as given in the section "Cell Lysis and Total Protein Determination." Protein samples were incubated with the reagent for 2 hours at 37°C in the dark, followed by measuring the absorbance of pNA (p-nitroaniline) released at the end of the reaction at 405 nm using a microplate reader at the 0, 1st and the 2nd hours as triplicates, while untreated cells were used as control. Results were expressed as caspase activity (pNA/ protein).

2.6. Flow Cytometry Analysis

Qifikit (fluorometric bead solution for quantitation) kit (Cat no:K0078, DAKO, Agilent, CA, USA) was used for receptor quantification. 100 µL of both set-up and calibration beads were transferred to separate flow cytometry tubes, washed once with PBS w/o Ca^{2+} and Mg^{2+} containing 0.1% sodium azide by centrifuging at 1800 rpm for 5 minutes. Supernatants were discarded, tubes were vortexed and beads were labelled with 10 µL fluorescein isothiocyanide (FITC) secondary antibody (1/50 diluted in PBS) by incubating for 1 hour at +4°C. HT-29 cells were detached by trypsinization, cells were pelleted by centrifuging at 1800 rpm for 5 minutes and counted by JuLi Br&Fl Station (NanoEnTek, South Korea). Cell concentration was adjusted to $2x10^5$ per mL. Cells were labelled with anti-stabilin-1 (1:500/tube) and anti-integrin αvβ3 (7 µL/tube) receptor antibodies by incubating cells at +4°C. All measurements were performed as triplicates. Tubes were read with Beckman Coulter CytoFLEX flow cytometry system (IN, USA), and analyses were performed with CytExpert software.

For receptor quantification, bead population was gated from the Side Scatter/Forward Scatter dot plot. Mean fluorescence intensities (MFI) regarding each bead population were obtained according to the respective histogram plot. For calculating stabilin-1 and integrin αvβ3 receptors on HT-29 cells, quantitative antibody binding capacity (ABC) was calculated according to samples' respective MFI values; the measured MFI value for each sample was compared with the respective bead MFI value which is the closest. Receptor numbers (BAE) of unstained control cells were calculated in the same manner, and specific antibody binding capacity (SABC), the quantitative antibody numbers, were calculated for each group by subtracting the ABC values of the samples.

2.7. Statistical Analysis

GraphPad Prism5 software was used to perform the statistical analysis. Statistical analysis was performed using one-way ANOVA (pairwise comparison test: Tukey) and two-way ANOVA test (pairwise comparison test: Bonferroni) with significances set at *ns: p>0.05,* *p < 0.05, ** p < 0.01 and *** p < 0.001 as indicated.

3. RESULTS

3.1. Cytotoxicity Analysis by xCELLigence System

IC_{50} values for SPARC and cilengitide was calculated according to the data obtained from xCELLigence real-time cell analysis systems; and IC_{50} values of these two molecules were determined as $4.57463x10^{-6}$ g/mL (Figure 1) and 50 nM (Figure 2), respectively.

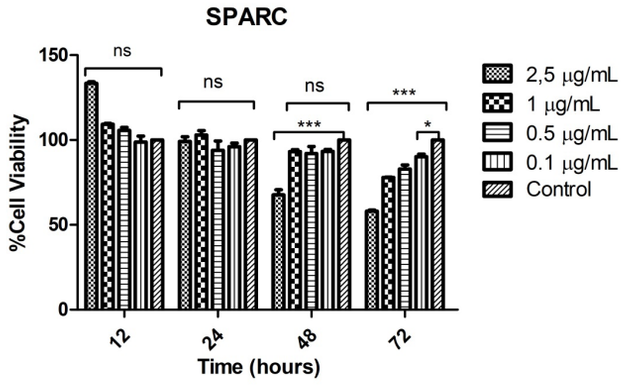

Figure 1**. IC_{50} values for SPARC protein on HT-29 cells (p<0.001, *p<0.05, ns:p>0.05, relative to control)*

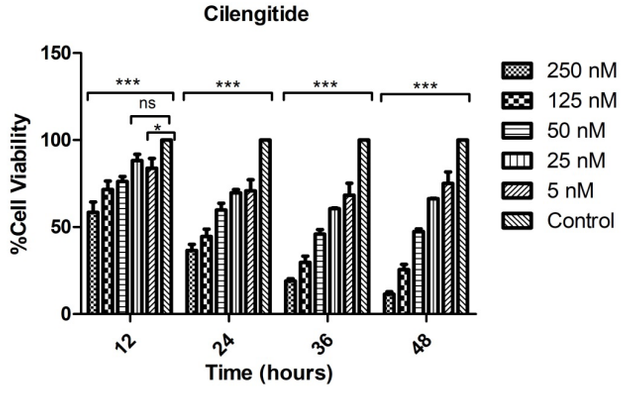

Figure 2**. IC_{50} values for cilengitide molecule on HT-29 cells (p<0.001, *p<0.05, ns:p>0.05, relative to control)*

3.2. Caspase-3 Levels

Caspase-3 enzyme activity in HT-29 cells was measured after incubating cells with either 5µg/mL SPARC or 5µg/mL SPARC and cilengitide simultaneously for two hours while untreated cells were used as control (Figure 3). 5 µg/mL SPARC significantly increased caspase-3 activity in all timepoints in comparison to the control group (p<0.001) while combinatorial treatment with SPARC and cilengitide significantly decreased active caspase-3 levels (p<0.001), indicating exogenous SPARC is a promoter of caspase-3

mediated apoptosis, and its pharmacological inhibition reverses the apoptotic effect.

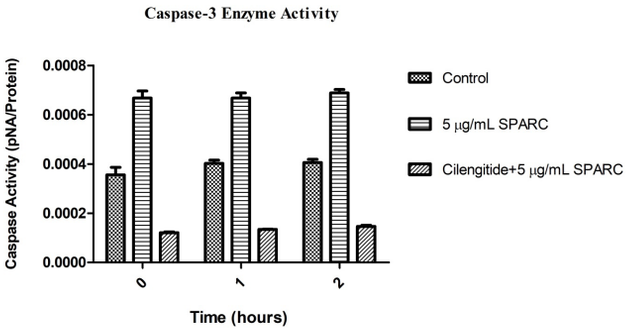

***Figure 3**. Caspase-3 activity results measured at 0, 1 and 2 hours in groups incubated with substances for 24 hours (p<0.001)*

3.3. RT-PCR Results

According to Figure 4a, when the gene expressions of SPARC, stabilin-1 and integrins in HT-29 cells were examined, it was determined that the base expressions of SPARC and integrins were quite low, but the expressions of stabilin-1 were higher. When 5µg/mL SPARC was added externally to the same cells, statistically significant differences were observed between SPARC, integrin αv and β3 expressions compared to the control ($p<0.001$, $p<0.001$ respectively). At the level of stabilin-1, there was no statistically significant difference ($p>0.05$).

As seen from the previous Figure 4a, the initial expressions of integrin αv and β3 were quite low and increased significantly with 5 µg/mL SPARC. However, when the integrin inhibitor cilengitide was added to the HT-29 cell lines first and then treated with 5 µg/mL SPARC, the gene expressions of both integrins were significantly reduced ($p<0.001$ and $p<0.01$, respectively) (Figure 4b).

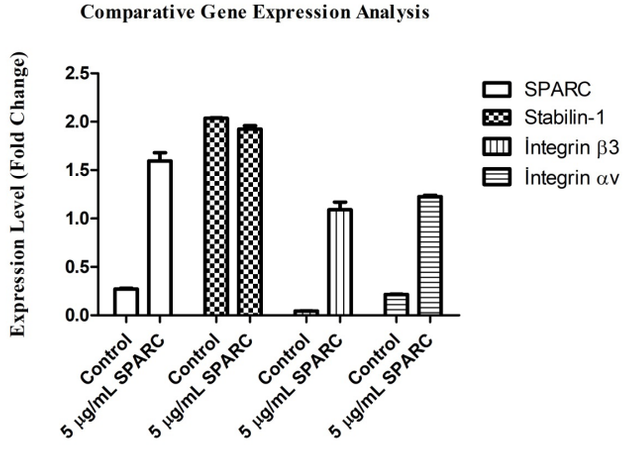

***Figure 4a**. Expression levels of SPARC, stabilin-1, integrin αv and β3 genes in HT-29 cells of control (base) and with 5µg/mL SPARC (terms of fold increase)*

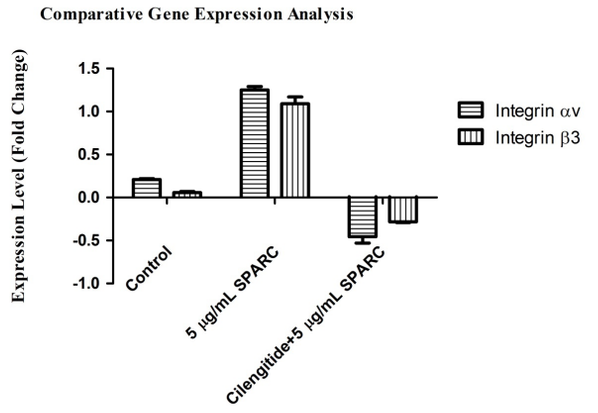

***Figure 4b**. Expression levels of integrin αv and β3 genes in HT-29 cells with 5µg/mL SPARC and cilengitide+5µg/mL SPARC (terms of fold increase)*

3.4. Flow Cytometry Results

The mean number of integrin αvβ3 receptors in the HT-29 cell line in the control group was 34203±9790/cell while addition of 5 µg/mL SPARC increased integrin αvβ3 receptor count to 74496±1665/cell, leading to a significant difference ($p<0.01$). However, cilengitide addition decreased integrin αvβ3 receptor count to 25731±12442, which is significantly lower than both control and 5 µg/mL SPARC groups ($p<0.01$) (Figure 6). When considering stabilin-1 receptor count; no significant difference between control and 5 µg/mL SPARC groups was observed ($p>0.05$) (Figure 7). The histogram plot, indicating five different bead population is given in Figure 5.

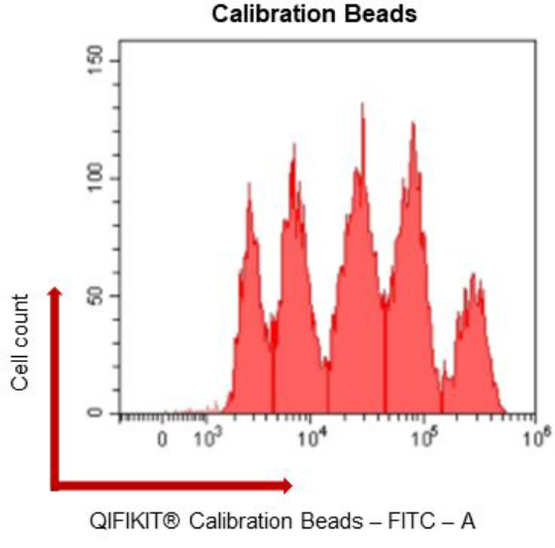

***Figure 5**. Histogram of calibrator beads used to determine quantitative receptor numbers*

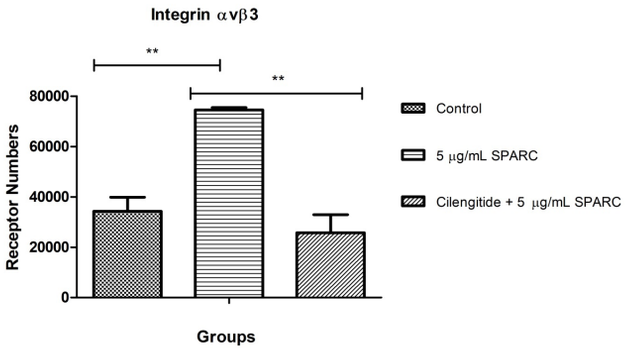

***Figure 6**. Integrin αvβ3 receptors numbers in different groups (**p<0.01)*

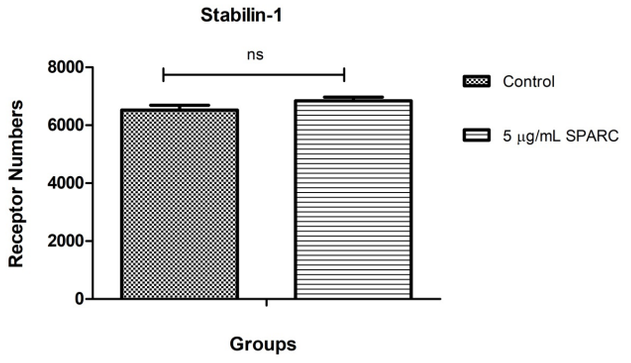

***Figure 7**. Stabilin-1 receptor numbers different groups (ns: p>0.05)*

4. DISCUSSION

In this study, firstly we determined SPARC protein, integrin αvβ3, and stabilin-1 levels in HT-29 cells, a colon cancer cell line. Then, the effects of exogenous SPARC protein at different concentrations on its possible receptors integrin αvβ3 and stabilin-1 in colon cancer cells were investigated. During this research, the cilengitide molecule, an integrin inhibitor, was used.

In recent studies, it has been found that SPARC has many roles on important molecular mechanisms in malignancies, including tumor microenvironment, apoptosis, and is effective in the regulation of tumor growth/invasion.

The findings suggest that SPARC may perform a tissue-specific function in cancer development, with SPARC being downregulated in some cancers and upregulated in other cancers. Studies in lines with colon cancer show that downregulation of SPARC is associated with aberrant methylation, particularly in the promoter region, thus suppressing methylation of CpG islands in the presence of SPARC. Despite the fact that most studies in people with colon cancer show that high SPARC expression is associated with a better disease outcome and low expression is associated with a worse prognosis, studies on SPARC expression and its relationship with pathological features in this cancer are extremely rare, and its prognostic value is still debated.

Our first findings by western blot analysis show that, in agreement with the literature, SPARC expression is greatly reduced in colon cancer lines (data not shown), these values are more clearly demonstrated as gene levels measured by RT-PCR in our study (as observed in Fig4a).

There is very little research in the literature on the interactions between tumor cell and exogenous SPARC. According to a study conducted on a prostate cancer cell line, it has been shown that the externally added SPARC protein causes inhibition in cell proliferation dose dependently (20, 21). Other study on the SPARC protein has shown that increased expression in neuroblastoma cells suppresses the proliferation of cells.

In the light of the information from the literature, in our xCELLigence system results, it was observed that SPARC suppressed cell proliferation at increasing concentrations that we added externally, and the most effective dose was found to be IC_{50}: 4.57 µg/mL (Figure 1).

In SKOV3 cells (The ovarian carcinoma cell line), the addition of 5 µg/mL and 20 µg/mL SPARC reduced the number of living cells to approximately 68% and 54% of the control respectively (22).

In the literature, it is seen that SPARC has different behaviors related to apoptosis in different cancer types. In a study on melanoma cells, an increase in the activity of caspase enzymes was observed after SPARC knockdown, and in another study on human ovarian cancer, high expression of SPARC protein was shown to indicate a poor prognosis mediated by apoptosis (23, 24). Our results with the apoptotic enzyme caspase-3 activity support our xCELLigence system results. We observed that the enzyme activity increased in the groups to which SPARC was added. In a study in colorectal cancer cell lines, SPARC addition during chemotherapy treatment further potentiated apoptosis (25).

Paralelly, according to our results, the increase of SPARC in the colon cancer cells induced apoptosis. Unlike other studies, when we used cilengitide, an integrin inhibitor, the apoptotic effect of SPARC was reduced. This case seems to be very supportive for the next step data in which we investigated the possible receptors of SPARC in colon cancer cells in our study.

The cell surface receptors of SPARC remain unclear, however, it can be said to act as a competitor of ligand-receptor interactions. In two separate studies, it was determined that integrin β4 controls the SPARC protein to stimulate invasion in breast cancer, whereas SPARC induces invasion in melanoma via a collagen I/α2β1 integrin pathway (26, 27).

In our study, two receptors were preferred according to the literature to elucidate the interaction of the exogenous SPARC protein, which showed antiproliferative effect on colon cancer cell line, with possible receptors. These were selected as integrin αvβ3 and stabilin-1 (7, 21).

According to our RT-PCR observations (Figure 4b), the baseline values of the subunits of the integrin αvβ3 receptor in colon cancer lines were quite low, as were the SPARC protein values. However, stabilin-1 receptor expressions, which are known to be found in macrophages and some endothelial cells, were found to be quite high (28). Moreover, exogenous SPARC protein significantly increased the expression of integrin αvβ3 subunits, while stabilin-1 receptor expressions remained at the same levels as control ($p>0.05$).

SPARC has been demonstrated to decrease adhesion, invasion, and cell proliferation in human ovarian cancer cells by lowering the surface localization and/or aggregation of αv, β1, β3 and β5 integrins, according to a study (28-30).

Since SPARC affects integrin interaction in many different cell types and this interaction is known to be cell type dependent, we determined that with increasing SPARC concentration (5 µg/mL), integrin response in colon cancer cells increased both at the gene level and quantitatively in terms of receptor numbers (base αvβ3 receptor numbers: 34203±9790/per cell, after 5 µg/mL SPARC, αvβ3 receptor numbers: 74496±1665/ per cell).

Incubation of colon cancer cells with the integrin inhibitor cilengitide first, significantly reduced integrin levels (to less than the control values) by inhibiting binding of receptor antibodies (Fig4b, Fig6, Table 1) (after 5 µg/mL SPARC, integrine αvβ3 receptor numbers: 74496±1665/ per cell and; after cilengitide+5µg/mL SPARC, αvβ3 receptor numbers: 25731±12442/ per cell).

Our RT-PCR and flow cytometric findings show that the possible receptor of SPARC in colon cancer is likely to be integrin αvβ3 receptor and intracellular transport is mostly via this receptor (integrin αvβ3 receptor), but the same is not true for stabilin-1. Even more to the best of our knowledge, in this study, we determined the number of integrin receptors per cell, especially in colon cancer cells, for the first time in the literature.

Table 1. *Receptor numbers of Integrin αvβ3 and Stabilin-1*

	İntegrin αvβ3	Stabilin-1
Control	34203±9790/cell	6525± 287.6/cell
5 µg/mL SPARC	74496±1665 /cell	6845± 220.3/cell
Cilengitide+5 µg/mL SPARC	25731±12442 /cell	-

5. CONCLUSION

In this study, we showed that exogenous SPARC can reduce proliferation and induce apoptosis in colon cancer cells. Although different signaling pathways mediate these tumor suppressor effects, they are likely dependent on SPARC binding to its cell surface receptor. The interaction of SPARC and its putative receptor may also contribute to the tissue- and cell-specific biological functions of SPARC in different normal and cancerous cells. According to our results, this possible receptor integrin αvβ3 may be in colon cancer cells, and the use of inhibitors strongly supports this interpretation. Quantification of receptors on the cancer cell surface can be considered as a marker in the follow-up of anticancer treatments. However, further studies are needed in this regard.

Funding: *This work was supported by the Research Fund of the University of Marmara (SAG-C-DRP 131.216.0536).*
Conflicts of interest: *The authors declare that they have no conflict of interest.*
Peer-review: *Externally peer-reviewed.*
Author Contributions:
Research idea: DÖ, ÖBÖ
Design of the study: DÖ, ÖBÖ
Acquisition of data for the study: DM
Analysis of data for the study: DM, TŞ, BA
Interpretation of data for the study: DM, TŞ, ST
Drafting the manuscript: DÖ, DM
Revising it critically for important intellectual content: DÖ, ÖBÖ, GYD
Final approval of the version to be published: DÖ

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How to cite this article: Mısırlı D, Bingöl Özakpınar O, Şekerler T, Aru B, Yanıkkaya Demirel G, Tunoğlu S, Özsavcı D. Effects of SPARC and Possible Receptors on Colon Cancer Cell Line. Clin Exp Health Sci 2023; 13: 316-322. DOI: 10.33808/clinexphealthsci.1100770