Effects of Type I Collagen and Fibronectin on Regulation of Breast Cancer Cell Biological and Biomechanical Characteristics

Yanqun Teng$^{1,2}$ Juhui Qiu$^{1,2}$ Yiming Zheng$^1$ Xiangdong Luo$^{2,4}$ Linlin Zhang$^{2,4}$

Li Chen$^{2,3,4,*}$ Guixue Wang$^1,*$

$^1$Key Laboratory of Bio rheological Science and Technology, Ministry of Education, Chongqing Engineering Laboratory in Vascular Implants, Bioengineering College of Chongqing University, Chongqing 400044, China
$^2$Burn Research Institute, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
$^3$Breast Disease Center, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
$^4$National Key Laboratory of Trauma and Burns, Chongqing Key Laboratory of Disease Proteomics, Chongqing 400044, China

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Abstract

Type I collagen (Col I) and fibronectin (FN) are important matrix proteins. Their biological roles in breast cancer are still unknown. This study investigates the effects of Col I and FN on the biological and biomechanical characteristics of breast cancer cells. Breast cancer MDA-MB 231 and MCF-7 cell lines were cultured on Col-I- or FN-coated plates or coverslips. Cell adhesion, spreading, proliferation, migration, and elasticity assays were performed. Changes in proliferation and migration were observed after using antibodies inhibiting syndecan-1 (syn-1) or syndecan-4 (syn-4). It was found that Col I promoted cell adhesion and spreading, and induced cytoskeleton organization of the two types of breast cancer cell, especially MDA-MB 231 cells. FN promoted breast tumor cell proliferation. Atomic force microscopy data show that the Young’s modulus of MDA-MB 231 (MCF-7) cells was much lower (higher) on Col I than on FN. After incubation with syn-1 or syn-4 antibodies, FN-induced cell proliferation was inhibited. These data demonstrate that Col I and FN have different effects on the biological and biomechanical characteristics of breast cancer cells, with syn-1 and syn-4 involved in FN-regulated proliferation.

Keywords: Breast cancer, Type I collagen (Col I), Fibronectin (FN), Cell elasticity, Syndecans

1. Introduction

Breast cancer is the most common invasive cancer among women in the world [1]. Despite recent advances in its early detection, prevention, and treatment, breast cancer is still the leading cause of cancer death annually in women worldwide due to frequent tumor metastasis, which makes it almost incurable [2]. Therefore, further understanding of the underlying molecular mechanisms responsible for mammary gland carcinogenesis could provide novel strategies for breast cancer prevention and treatment.

The extracellular matrix (ECM) provides structural and mechanical supporting networks for cells and tissues in addition to other important functions in tissues. The ECM plays a critical role in cell proliferation, adhesion, and migration [3]. During tumorigenesis, the ECM is part of the tumor microenvironment, supporting tumor cell growth and progression [4]. The ECM consists of collagens, glycoproteins, and glycosaminoglycans. The changes in its composition alter cancer cell growth, invasion, and metastasis [5,6]. Among these ECM components, type I collagen (Col I) is always expressed in breast cancer stroma, and is increased in the interstitial matrix of neoplastic breast tissue [6]. Fibronectin (FN) is strongly expressed in breast carcinoma, and its distribution is different from that of normal breast parenchyma [7,8], suggesting that these two ECM components are important regulators in breast cancer development and progression. However, the mechanism underlying their regulation of tumorigenesis through cellular behaviors remains unclear.

It has been shown that ECM proteins not only influence the cellular biological properties, but also change the cell stiffness of cancer cells [9]. It has been reported that cellular softening (decrease in stiffness) is linked to the malignancy and...
metastasis of tumor cells [10-12]. There is growing evidence showing that a minor change in cell stiffness can be regarded as a harbinger of some diseases [4,11]. Atomic force microscopy (AFM) has been used to analyze cellular elasticity associated with carcinogenesis [11].

The present study determines the role of these ECM components and the possible mechanisms of their effects on the biological properties and cell stiffness of different breast cancer cells. The roles of FN and Col I in the regulation of breast cancer cell adhesion and spreading are first investigated. Then, the proliferation and migration capacity of breast cancer cells cultured on Col-I- and FN-coated plates are analyzed. The effects of syndecan-1 (syn-1) and syndecan-4 (syn-4) on cell proliferation and migration regulated by ECM proteins are evaluated since syndecans have been reported to bind to various ECM proteins, such as FN and Col I [13], and to be critical in regulating tumor cell adhesion and invasion [14]. Finally, the effects of Col I and FN on cell elasticity are investigated using AFM. The data demonstrate that FN and Col I play important roles in breast cancer cell adhesion, spreading, proliferation, migration, and elasticity, and that syn-1 and syn-4 antibodies suppressed FN-induced proliferation.

2. Materials and methods

2.1 Cell lines and culture

Human breast cancer cell lines MCF-7 and MDA-MB 231 were purchased from American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (Invitrogen Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). MDA-MB 231 was cultured in Roswell Park Memorial Institute (RPMI)-1640 (Invitrogen-Gibco, NY, USA) supplemented with 10% FBS in a humidified incubator with 5% CO₂ at 37 °C. For the experiments, all tissue culture plates or coverslips were pre-coated with 10 µg/ml Col I (BD Biosciences, San Jose, CA, USA) or human FN (BD Biosciences) for 2 hours at 37 °C [15], and then washed thoroughly with phosphate-buffered saline (PBS).

2.2 Tumor cell adhesion and spreading assay

Cells were harvested and resuspended in serum-free medium at 1 × 10⁶ cells/ml and 250 µl of cell suspension was seeded onto each well of 24-well plates (Nunc, Roskilde, Denmark) that were pre-coated with 10 µg/ml Col I or FN. After the plates were incubated for 15, 30, and 60 min, unadherent cells were removed by washing with PBS. The adherent cells were trypsinized and counted with a haemocytometer [16].

To assess the effect of Col I and FN on cell spreading, MCF-7 and MDA-MB 231 cells were inoculated on pre-coated glass coverslips, which were placed in a 24-well plate. 5 × 10⁵ cells were added to each well. After 4 or 12 h of incubation, cell spreading was imaged by phase-contrast microscopy (OLYMPUS IX 81, Olympus, Tokyo, Japan), and the spreading area of a single cell was measured by Image Tool software (The University of Texas Health Science Center at San Antonio, Texas, USA). All the data were obtained from at least three parallel wells and repeated in three independent experiments.

2.3 F-actin staining

MDA-MB 231 and MCF-7 cells were inoculated on pre-coated glass coverslips for approximately 24 h. The cells were then washed thrice with PBS, fixed in 3.7% paraformaldehyde for 15 min, and incubated with 1% BSA for 1 h at room temperature. F-actin was stained with FITC-Phalloidin (Alexis, San Diego, CA, USA) for 1 h. After being rinsed thoroughly with PBS, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Nantong, Jiangsu, China) for 5 min. After being washed three times with PBS, the coverslips were reviewed and microphotographed using an inverted fluorescence microscope (OLYMPUS IX 81).

2.4 Cell viability assay

To assess the effects of Col I or FN on the regulation of breast cancer cell growth, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2h-tetrazolium bromide (MTT) assay was performed to determine cell viability. Briefly, a total of 1×10⁴ cells per well were plated in triplicate in pre-coated 96-well plates. After incubation for 1 to 5 days, MTT solution (5 mg/ml in PBS; ICN Biomedicals, Costa Mesa, CA, USA) was added to the plates and incubated for 4 h. Subsequently, the solution was carefully discarded and 100 µl of dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA) was added to each well. The absorbance rate was measured at a wavelength of 490 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). This assay was conducted in three independent experiments.

Syndecans are crucial in the regulation of cell proliferation and migration [17,18]. To determine the effects of syn-1 and syn-4 on ECM proteins-regulated breast cancer cell proliferation, 1 µg/ml syn-1 and syn-4 antibodies were added into the cell culture after the cells were normally inoculated for 24 h, and continuously cultured for an additional three days followed by the MTT assay. Both anti-syn-1 and anti-syn-4 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The efficacy of the anti-syn-1 and anti-syn-4 concentration (1 µg/ml) used in the experiments was tested (data not shown).

2.5 Tumor cell wound healing assay

Cells were cultured to 80% confluence, and then incubated overnight in medium containing 2% FBS. Next, the cells were treated with 6 µg/ml mitomycin C (Sigma, Steinheim, Germany) for 2 h, and washed thoroughly with PBS. The efficacy of mitomycin C concentration and time was tested (data not shown). Then, a monolayer was scratched using a 200-µl pipette and washed three times with PBS to remove loose cells, and then cultured up to 12 h with medium containing 2% FBS. Images were obtained using phase-contrast microscopy at 0, 3, 6, and 12 h. The wound area was measured using Image Tool software, and the tumor cell migration rate (MR) was
calculated as:

\[ \text{MR} (\%) = (1 - A_t / A_0) \times 100\% \]  

(1)

where \( A_0 \) and \( A_t \) represent the wound areas at the initial time and \( t \), respectively. All the results were obtained from three parallel wells from three separate experiments.

This assay was repeated after cells were pretreated with 1 \( \mu \)g/ml anti-syn-1 and anti-syn-4 for 12 h. Syn-1 and syn-4 antibodies were added to the experimental wells afterwounding.

2.6 Cell elasticity measurement by atomic force microscopy

Cells were seeded on coverslips (22 mm in diameter) and incubated for 24 h. The elasticity measurements were performed on a JPK Nanowizard II atomic force microscope (JPK Instruments AG Berlin, Germany). An environmental cell control was employed to control the temperature of the solution above the coverslips. Briefly, silicon nitride AFM cantilevers (Olympus Optical, Co., Ltd., Tokyo, Japan) were used for Young’s modulus measurements. The probe tip (height = 2.9 \( \mu \)m) is shown in the top right. Scale bar = 25 \( \mu \)m. (a) Silicon nitride AFM cantilevers were used for Young’s modulus measurements. The force curves were imported into JPK Image Processing software (Version 3.3.25; JPK Instruments AG) to calculate the Young’s modulus based on the Hertz model [19]:

\[ F = \delta^2 \frac{2}{\pi} \frac{E}{(1 - v^2)} \tan(\alpha) \]  

(2)

where \( F \) is the indentation force, \( \delta \) is the indentation depth, \( v \) is the Poisson’s ratio, \( \alpha \) is the half-opening angle of the AFM cantilever, and \( E \) is the Young’s modulus to be determined. The cell was assumed to be incompressible and Poisson’s ratio = 0.5 [20].

![AFM indentation for single cells.](image)

(a) (b)

Figure 1. AFM indentation for single cells. (a) Silicon nitride AFM cantilevers were used for Young’s modulus measurements. The probe tip (height = 2.9 \( \mu \)m) is shown in the top right. Scale bar = 25 \( \mu \)m. (b) Screenshot of AFM indentation experiments. The blue pane is the AFM scan field (100 \( \times \) 100 \( \mu \)m²). The Arabic numerals in the vicinity of the nuclei represent indentation points randomly selected for each cell.

2.7 Statistical analysis

Data are summarized as mean ± standard error (SE) from at least three independent experiments. Statistical analysis was performed using SPSS statistical software v17.0 (SPSS, Chicago, IL, USA) for one-way analysis of variance (ANOVA). A \( p \) value of < 0.05 was considered statistically significant.

3. Results

3.1 Effect of type I collagen on breast cancer cell adhesion and spreading

Cell adhesion and spreading are essential for cancer metastasis, so it was first determined whether the ECM components had different effects on the regulation of breast cancer cell morphology, adhesion, and spreading. Changes in the cellular morphology of breast cancer cells cultivated on Col-I- or FN-coated glass coverslips were observed. MDA-MB 231 cells cultivated on Col-I-coated coverslips showed a larger cell body and longer pseudopodium structures compared to those of cells cultured on FN (Fig. 2(a)). MCF-7 cells did not show any changes in the cellular morphology when cultured on these two ECM-protein-coated coverslips. The spreading of these two cell lines was then assessed. It was found that Col-I significantly promoted the spreading of MDA-MB 231 cells at 4 and 12 h compared to that done by FN (Fig. 2(b)). The spreading area of MCF-7 cells increased only at 4 h. Next, cells adhesion was examined. The data show that MCF-7 and MDA-MB 231 cells tended to attach onto Col-I-coated plates within 30 min (Fig. 2(c)), 15 min after seeding, the number of adherent MDA-MB 231 cells on Col-I-coated plates was 7-fold that on FN. F-actin staining shows that the two cell lines, and especially MDA-MB 231, had a more pronounced and well-aligned network of actin fibers when cultured on Col-I and that these actin filaments were parallel in the cytoplasm (Fig. 2(d)).

3.2 Effect of fibronectin on breast cancer cell proliferation

The proliferation and migration of cancer cells are critical processes during cancer development. It was thus determined how the ECM components regulated the breast cancer cell proliferation and migration. The MTT assay results show that FN significantly promoted the proliferation of MDA-MB 231 cells compared to Col-I (Fig. 3(a); \( p < 0.05 \)). Similarly, FN induced MCF-7 cell proliferation. The cell wound healing assay results show that FN promoted the migration of both cell lines, but it was not statistically significant (Fig. 3(b)).

3.3 Effects of syndecan-1 and syndecan-4 antibodies on FN-induced tumor cell proliferation

In breast cancer, the overexpression of syn-1 is associated with poor prognosis and an aggressive phenotype, and syn-4 has been reported to be expressed in most breast carcinoma cell
Figure 2. Effects of Col I and FN on cellular morphology, spreading, adhesion, and F-actin filament structure. (a) Changed tumor cell morphology. MDA-MB 231 and MCF-7 cells were seeded and grown on Col-I- or FN-coated coverslips for 12 h and the cells were then photographed without washing. Scale bar = 80 μm. (b) Cell spreading capacity. Col I improved cell spreading capacity in both tumor cell lines, especially in MDA-MB 231 cells. (data are summarized as mean ± SE; * p < 0.05, # p < 0.01). (c) Tumor cell adhesion properties. Cells were seeded in pre-coated plates and incubated up to 60 min. Unadherent cells were removed and the adherent cells were counted by a haemocytometer (*and ** p < 0.05; # and ## p < 0.01, * and # represent MCF-7 cells grown on Col I vs. FN; ** and ## represent MDA-MB 231 cells grown on Col I vs. FN. Data are summarized as mean ± SE). (d) F-actin staining images of cellular cytoskeleton. F-actin filaments are shown in green and the cell nuclei in blue. Scale bar = 20 μm. Data were obtained from at least three independent experiments.

Figure 3. Effects of Col I and FN on tumor cell proliferation and migration. (a) FN promoted proliferation of MDA-MB 231 and MCF-7 cells. Both cells were seeded and grown in Col-I- or FN-coated 96-well plates for up to 5 days and then subjected to MTT assay. (* and ** p < 0.05; * indicates MCF-7 cells grown in Col-I-coated plates vs. FN-coated plates; ** indicates MDA-MB 231 cells). (b) Both MDA-MB 231 and MCF-7 cell lines were seeded and grown in Col-I- or FN-coated 12-well plates and subjected to wound healing assay. Scale bar = 400 μm. FN promoted the migration of both cell lines, but it was not statistically significant. The data are presented as mean ± SE from three independent experiments.

3.4 Effect of matrix proteins on tumor cell elasticity

Cell mechanical properties, such as elasticity and viscosity, play a fundamental role in cell growth [21]. Thus, the cell elasticity in response to matrix proteins was investigated. The elasticity of a single breast cancer cell was measured by AFM. The extended force curve was chosen for fitting and calculating the final result (Fig. 5(a)). The modulus of MDA-MB 231 cells on FN was as high as 2204 Pa, approximately 2-fold that on Col I (1106 Pa). In contrast, MCF-7 cells became significantly stiffer when grown on Col I matrix, with an average Young’s modulus of 1647 Pa, whereas the Young’s modulus was about 787 Pa when the cells were grown on FN (Fig. 5(b)).
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4. Discussion

The primary goal of this study was to investigate the effects of Col I and FN, two important ECM constituents, on the adhesion, spreading, proliferation, migration, and elasticity of breast cancer cell lines MDA-MB 231 and MCF-7. It was found that Col I and FN had different effects on the regulation of breast tumor cell adhesion, spreading, proliferation, migration, and elasticity. Col I improved cell morphology, adhesion, and spreading, whereas FN promoted cell proliferation. Moreover, syn-1 and syn-4 proteins were found to be involved in FN-induced breast cancer cell proliferation. In addition, Col I softened MDA-MB 231 cells; however, MCF-7 cells were stiffer on Col I than on FN.

A high expression of collagen is associated with elevated incidence of tumorigenesis and metastasis in mammary glands [22,23]. A previous study [24] has indicated that breast tumorigenesis is accompanied by Col I crosslinking, tissue elastic drifting, and increased cell adhesion. Our results show that the two types of breast cancer cell, and especially MDA-MB 231 cells, were prone to attach to Col I and exhibited...
a visibly larger spreading area on Col I than on FN (Figs. 2(b) and 2(c)). Moreover, F-actin filaments were well organized when cells were cultured on Col I (Fig. 2(d)). Adhesion and spreading play key roles in tumor metastasis, so these results suggest that Col I might contribute to the metastatic progression of breast cancer cells. Docheva et al. [9] reported that prostate cancer PC3 cells adhered more strongly on Col I than on FN, which may indicate that PC3 cells have high bone metastatic potential.

FN is highly expressed in breast tumors. Ioachimia et al. reported that a high level of FN expression is positively correlated with lymph node involvement and the proliferation associated index Ki-67 [25]. They also found an increased mortality risk associated with the high level of FN expression by survival analysis. The data presented here show that FN promoted the proliferation of both MDA-MB-231 and MCF-7 cells (Fig. 3(a)). Previous studies found that the proliferation of mammary epithelial cells was promoted by FN in three dimensional culture [26]. Thus, our results suggest that FN is important for the hyperplasia of neoplastic tissues during breast cancer development and progression. Interestingly, weak binding to the ECM is correlated with enhanced proliferation of tumor cells [27] which could partly explain why FN promotes the proliferation of MDA-MB-231 and MCF-7 cells.

Syndecans are important cell surface proteoglycans that can bind to a number of different ECM ligands [28]. This study thus examined whether syndecans were involved in FN-induced cancer cell proliferation and migration. Our data suggest that syn-1 and syn-4 proteins are involved in regulating proliferation in response to FN (Fig. 4(a)). Syn-1 and especially syn-4 induced tumor development at least in part through the upregulation of cell proliferation on FN. Increased syn-1 has been shown to be associated with tumor development and poor prognosis of breast cancer and modulated tumor cell proliferation and migration [18,29,30]. It has also been reported that syn-4 restores focal adhesion kinase (FAK) phosphorylation on the FN in tumor cells [31]. FAK plays a central role in the regulation of the proliferation and migration of tumor cells [32]. According to our results and previous reports, syn-1 and syn-4, especially syn-4, may act as a link between breast tumorigenesis and the change of ECM protein levels in cancer tissues.

It has been reported that the elastic modulus of metastatic breast cancer cells is approximately 70% lower than that of normal cells from a given patient, and thus cell stiffness can serve as a potential biomarker to predict malignancy [11]. The present study found that the Young’s modulus of MDA-MB-231 cells ranged from 1 to 2.5 kPa and that of MCF-7 cells ranged from 0.7 to 1.8 kPa (Fig. 5(b)). Lower modulus values have been previously reported for these two cell lines (MDA-MB-231: 341 ± 41 Pa; MCF-7: 425 ± 31 Pa) [33]. This discrepancy may be due to the different experiment parameters applied, mainly the setpoint, indentation depth, and loading rate [12], but more likely due to the nonlinear stress-strain behavior of the cells or the mechanical properties of certain matrix proteins used. Cells are highly nonlinear and their elastic behavior depends on the degree of applied or internal stress [34] as well as the mechanical properties of their environment [35].

Our results show that Col I induced the adhesion and spreading of MDA-MB-231 cells (Figs. 2(b) and 2(c)), which play key roles in tumor metastasis, suggesting that Col I might contribute to the metastatic progression of breast cancer cells. On the other hand, AFM indentation results indicate that Col I induced a softening of MDA-MB-231 cells (Fig. 5(b)). Tumor cell softening is conducive to small deformation and could increase the mobility of malignant cells during tumor metastasis and invasion [36], suggesting a close relationship between tumor cellular biomechanical and biological characteristics. Collectively, our results suggest that Col I may promote breast cancer metastasis by softening tumor cells.

An interesting finding in our study is that MDA-MB 231 and MCF-7 cells had distinct stiffness responses to Col I and FN. Col I facilitated the softening of MDA-MB 231 cells, but not MCF-7 cells, compared to FN (Fig. 5(b)), leading to the hypothesis that MCF-7 cells, having lower invasive and metastatic potential than MDA-MB-231 cells [37], were not tightly regulated by Col-I-dependent softening changes. In other words, it is this difference of stiffness responses to Col I and FN might be lead to the different metastatic characteristics of the two cell types. It has been reported that higher cell stiffness is correlated with lower metastatic potential in certain cells [38], which is consistent with the relationship between lower metastatic potential and the Col-I-induced high stiffness of the MCF-7 cells. However, this hypothesis needs to be further verified. In addition, our results suggest that the two cell lines had distinct responses to ECM components. Therefore, the change of ECM components played a role in the regulation of the tumor cell biomechanical phenotype, which could influence tumor cell invasion and metastatic ability [38].

How the breast cancer cells orchestrate the biological and biomechanical behaviors in response to these two distinct matrix proteins requires further study that focuses on the triangular relationships among the tumor cell elasticity, biological properties (e.g. cytoskeletal reorganization), and the matrix proteins. Since syn-1 and syn-4 are important matrix-associated regulators of breast tumor cell proliferation and migration, an in-depth study is currently underway to reveal the effects of syn-1 and syn-4 on tumor cellular biomechanical properties in response to Col and FN matrix proteins.

5. Conclusion

Our study demonstrated that Col I and FN have different effects on the biological and biomechanical characteristics of breast cancer cells. Syn-1 and syn-4 involved in FN-regulated proliferation. Syn-1 and syn-4 antibodies, especially syn-4 antibody, inhibited FN-stimulated tumor cell proliferation.

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