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EFFECTS OF COLLAGEN TYPE 1 AND 3 ON CELL PROLIFERATION AND ALPHA FETO PROTEIN EXPRESSION IN A HUMAN CELL LINE MODEL OF LIVER CANCER

EKSTRASELLÜLER MATRİKS BİLEŞENLERİNDEN TİP 1 VE TİP 3 KOLLAJENİN KARACİĞER KANSERİ İNSAN HÜCRE HATTI MODELİNDE HÜCRE PROLİFERASYONUNA VE ALFA FETO PROTEİN EKSPRESYONU ÜZERİNE ETKİLERİ

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ABSTRACT

Objective: In cancer research, studies showing the behaviour of cells in different environments is important for developing new treatment methods. The microenvironment is essential for both regular and cancerous tissues. The main component of the extracellular matrix (ECM) is collagen. In HepG2 cells, a hepatocellular carcinoma cell line, the effects of Type 1 and Type 3 collagen on cell proliferation and the expression of alpha-feto protein (AFP), which is an indicator of carcinogenicity, was examined.

Material and Method: HepG2 cells were grown in Type 1 or Type 3 covered surfaces, whereas no treatment was applied to the control group. Proliferation analysis was performed via microscopic examination and cell viability assessmet kit (Cell Counting Kit-8, CCK-8). AFP was measured using a confocal microscope using immunological staining.

Result: The results showed that the viability rate of HepG2 cells growing in Type 3 collagen medium was statistically higher than in the control group ($p<0.0001$). AFP expression increased significantly in the presence of Type 3 collagen compared with the control group at the 24th hour ($p<0.05$), and at other culture times, AFP expression was seen more in Type 1 collagen culture medium.

ÖZET

Amaç: Kanser araştırmalarında, hücrelerin farklı ortamlardaki davranışlarını gösteren çalışmalar yeni tedavi yöntemlerinin geliştirilmesi için büyük önem taşımaktadır. Mikroçevre hem normal doku hem de kanserli doku için çok önemlidir. Ekstraselüler matriks (ECM) ana bileşenleri kollajenlerdir. Hepatosellüler karsinom hücre hattı olan HepG2 hücrelerinde Tip 1 ve Tip 3 kollajenin hücre proliferasyonu ve karsinojenite göstergesi olan alfa-feto protein (AFP) ekspresyonu üzerine etkisi incelenmiştir.

Gereç ve Yöntem: Proliferasyon analizi mikroskobik inceleme ve hücre canlılığı değerlendirme kiti (Cell Counting Kit-8, CCK-8) ile yapıldı. AFP ölçümleri immünolojik boyama yöntemleri kullanılarak konfokal mikroskop ile yapıldı.

Bulgular: Sonuçlar, Tip 3 kollajen ortamında büyüyen HepG2 hücrelerinin canlılık oranının kontrol grubuna göre istatistiksel olarak daha yüksek olduğunu gösterdi ($p<0,0001$). AFP ekspresyonu 24. saatte kontrol grubuna kıyasla Tip 3 kollajen varlığında anlamlı olarak artmış ($p<0,05$), diğer kültür zamanlarında ise AFP ekspresyonu Tip 1 kollajen kültür ortamında daha fazla görülmüştür.

Sonuç: Her iki tip kollajende kültüre edilen HepG2 hücrelerinin morfolojik olarak benzer bir yapıya sahip olduğu, Tip I kolla-

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Conclusion: HepG2 cells cultured in both types of collagen have a morphologically similar structure, and Type 1 and Type 3 collagen have a proliferation-enhancing effect on cancer cells. AFP, an indicator of liver cancer, is high in culture media containing Type 1 and Type 3 collagen and this finding is considered as the tendency of bad prognosis of collagen types on AFP expression.

Keywords: Alpha-fetoprotein, extracellular matrix, hepatocellular carcinoma, type 1 collagen, type 3 collagen

jen ve Tip 3 kollajenin kanser hücreleri üzerinde proliferasyonu artırıcı etkiye sahip olduğu belirlenmiştir. Karaciğer kanserinin göstergelerinden biri olan AFP, Tip 1 kollajen ve Tip 3 kollajen içeren kültür ortamlarında yüksek bulunmuş, bunun da kollajen tiplerinin AFP ekspresyonu üzerinde kötü prognoza doğru bir eğilim gösterdiğini düşündürmektedir.

Anahtar Kelimeler: Alfa fetoprotein, ekstrasellüler matriks, hepatosellüler karsinoma, tip 1 kollajen, tip 3 kollajen

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common solid tumour in incidence and third in mortality. Approximately 750,000 new cases are encountered each year. HCC causes 250,000 to 1 million deaths per year worldwide (1-4). Despite therapeutic advances, there has not been a significant improvement in the survival of patients with HCC in the last two decades. Systemic chemotherapy has not been shown to prolong survival in patients with HCC, and new approaches are critically needed to achieve a significant reduction in HCC mortality due to the failure of conventional chemotherapy. Therefore, the study of the molecular mechanism of HCC pathogenesis and the identification of new targets for molecular HCC therapy remain important (4, 5).

HepG2 cells were isolated from a 15-year-old patient diagnosed with HCC. They are frequently used to understand the mechanism of liver cancer and in therapeutic studies. Hepatoma cells are used to understand liver cancer phenotypes and the phenotypes of hepatocyte cells in healthy and diseased states (5-7).

In cancer research, studies showing the behaviour of cells in different environments is of great importance for developing new treatment methods. For cells to function normally, microenvironmental components must be in the required amount and order. The microenvironment is extremely important for both normal and cancerous tissues. One of the most important components of the microenvironment is extracellular matrix (ECM) components. The main component of the ECM is collagen (8).

When hepatocytes are cultured in vitro on flexible ECM-derived gels containing Type 1 collagen or basement membrane proteins, the culture substrate allows cells to assume different shapes and high expression of liver-specific genes. On the contrary, when hepatocytes are cultured on plastic surfaces, due to the in elasticity of the substrate, they show an undifferentiated, flattened shape, and there is a severe reduction the expression of liver-specific genes. Hepatocytes cultured in plastic or in a monolayer attached to ECM proteins such as collagen and laminin exhibit distorted morphology and liver-specific functions (9, 10). Molecular methods have shown that the level of Type 1 collagen is much higher in HCC samples than in normal liver samples (10). Type 3 collagen is the second most abundant collagen in the ECM (11).

In light of studies showing the in vitro and in vivo effects of different types of collagen in different cancer types, we aimed to examine how Type 1 and Type 3 collagen affect cell proliferation in HepG2 cells, a hepatocellular carcinoma cell line, and how collagen types affect the expression of alpha-feto protein, an indicator of carcinogenicity.

In short, we comparatively investigated the changes in the morphological and functional properties of HepG2 cells in the presence of Type 1 and Type 3 collagen compared with conventional cultured cells. The effect of collagen type on cell proliferation will be examined using a proliferation assay, and prognosis-related alpha feto protein (AFP) expression will be examined under a confocal microscope using immunohistochemical analysis.

MATERIAL AND METHODS

Cell culture

In the project; human hepatocellular cells obtained from ATCC (AmericanType Culture Collection, USA) carcinoma cell line Hep2cell. Cells were initially cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, UK) and 1% antibiotic (penicillin-streptomycin, Pan Bitech, Germany). Cells reaching 60-70% confluent were treated with trypsin (Gibco, UK), removed from the culture dish, counted, and divided into three main groups.

The cells were seeded into uncoated, Type 1 collagen-coated, or Type 3 collagen-coated in vitro culture plates. In the classical culture medium group, HepG2 cells were seeded in 24-well culture dishes with round coverslips at a density of 70,000 cells per well and 96-well culture dishes with 10,000 cells per well. These groups of cells were fed with routine cell culture medium, and proliferation and immunohistochemical analyses were performed at the indicated experimental times. Twenty-four and 96-well culture dishes coated with Type 1 collagen (Corning-354249, USA) were kept in the incubator for at least 4 h and washed with sterile phosphate buffered saline (PBS) before seeding. Culture dishes coated

with Type 3 collagen (Genlantis-Q3HCO100,Germany) were kept in an incubator for at least 4 h as in the control group. Proliferation rates and immunohistochemical analyses of these cells were performed at the same time points as in the conventional culture control group.

Monitoring of morphological changes

A- Microscopic analysis: To evaluate changes in the morphology of HepG2 cells, live images of the cells were recorded under an inverted phase microscope (Zeiss, Primovert, Germany) during the three periods mentioned above.

B- Haematoxylin-Eosin (H&E) Staining: Cells in two separate culture media seeded on sterile round coverslips in 24-well culture dishes were fixed with 4% formaldehyde (room temperature, 20 min.) at three different analysis periods. During fixation, PBS was applied thrice for 2 min. After fixation, the cells were stained with H&E. The nucleus structure and acidic and basic changes in the cytoplasm of the cells were evaluated after staining. The stained samples were examined and photographed under a light microscope at magnifications of 200 and 400X.

Proliferation assay

In our project, the proliferation of HepG2 cells was measured in three different groups and at three different times using a commercially available cell viability assesment kit (WST-8/CCK8, Ab228554, AbCam, UK). According to the kit procedure; 10 µL of CCK-8 solution was pipetted into 100 µL volume of cell suspension seeded in 96 wells on the days to be analysed, incubated in the incubator for 1-4 hours, and then spectrophotometrically read at 460 nm wavelength in a microplate reader (BioTek Synergy H1, US) and the colour change was evaluated with the optical densities obtained.

Immunohistochemical analysis

AFP immunofluorescence (IF) staining method:

Fixed HepG2 cells for AFP staining were maintained at 4°C until immunohistochemical staining. The coverslips in the fixative were first washed with Cello-IF solution and heated to 37°C for 3x5 min. The primary AFP antibody (Thermo, US) was prepared by diluting 1/100 with Cello-IF solution, treating cells, and incubating at 37°C. Cells were then washed with Cello-IF solution and heated to 37°C for 3x5 minutes. The secondary goat anti-rabbit IgG (H+L) Dylight 488, ThermoScientific) (diluted 1/200 with Cello-IF) specific for the primary antibody was incubated at 37°C. After incubation, cells were washed with warm PBS for 3x5 minutes and covered with Hoescht core stain (33258, Sigma, Germany) + Glycerol. The preparations were kept in the dark and at 4°C until examination using a confocal microscope (Zeiss, LSM700, Germany).

Statistical analysis

The data obtained from the study were evaluated using GraphPad Prism 9 analysis programme (GraphPad Prism V. 9.01). Two-way analysis of variance (ANOVA) was used to compare the results of proliferation measurements and AFP expression in three different culture media. Statistical significance was set as $p<0.05$.

RESULTS

Morphological analysis results

HepG2 cells were examined under an inverted microscope. In the culture medium supplemented with Type 1 collagen, it was observed that the cells had a spindle structure that differed from the classical polygonal cell form, and communication between cells increased through spindle extensions. It was observed that the cells in the culture medium containing Type 3 collagen exhibited similar morphological features and the cells had spindle-like extensions. Figure 1 shows live microscopy images of the cells.

Our experimental groups were analysed morphologically using H&E staining. Accordingly, the stained preparations were examined under a light microscope, and it was shown that the control group of HepG2 cells showed the expected morphology after staining the nucleus and cytoplasm separately. The spindle-like structure of HepG2 cells in Type 1 collagen medium was clearly observed by H&E staining, and a similar morphologic structure was observed in HepG2 cells in culture medium supplemented with Type 3 collagen. It was noticed that the cytoplasm of the cells had become larger and granularized. Figure 2 shows microscope images of the cells after H&E staining.

Proliferation outcome

In order to investigate the proliferation rate of HepG2 cells in different microenvironments, spectrophotometric readings of the cells were statistically analysed in proportion to viable cells using WST-8 as described in the methods section. Accordingly, at 24 h, the rate of viable cells in media containing Type 1 and Type 3 collagen showed a statistically significant increase compared with HepG2 cells in conventional culture media ($p<0.0001$). At the 48th hour, there was an increase in the number of viable cells in all cell groups compared with the first day, and the proliferation rates of the cells in the presence of collagen were higher than those of the control group cells, whereas the increase in HepG2 cells growing in medium with Type 3 collagen was statistically significant ($p<0.0001$). At 72 hours of the culture period, it was observed that the proliferation rate in all cell groups generally decreased compared with the previous day, the proliferation rate of the cells in the collagenous medium was higher than that in the control group, and statistically, the cells in the Type 3 collagenous medium showed a significant increase compared with the control group ($p<0.01$). Figure 3 shows the results of the CCK-8 test.

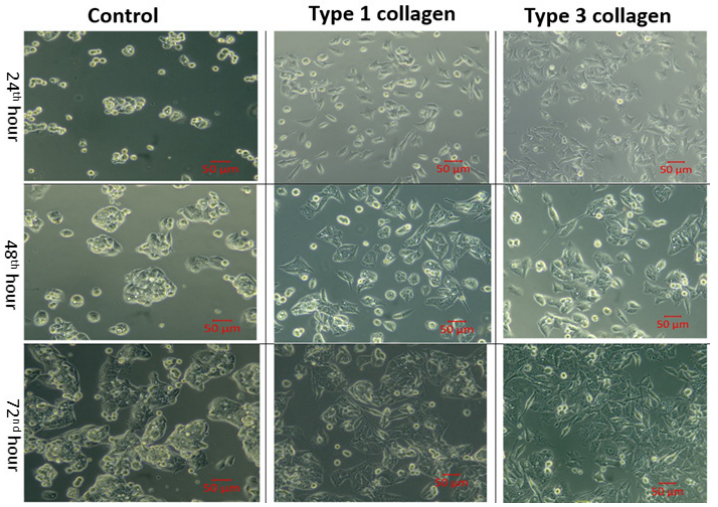

Figure 1: Live images of HepG2 cells under an inverted microscope in three different culture media and during three different culture periods. 20X magnification

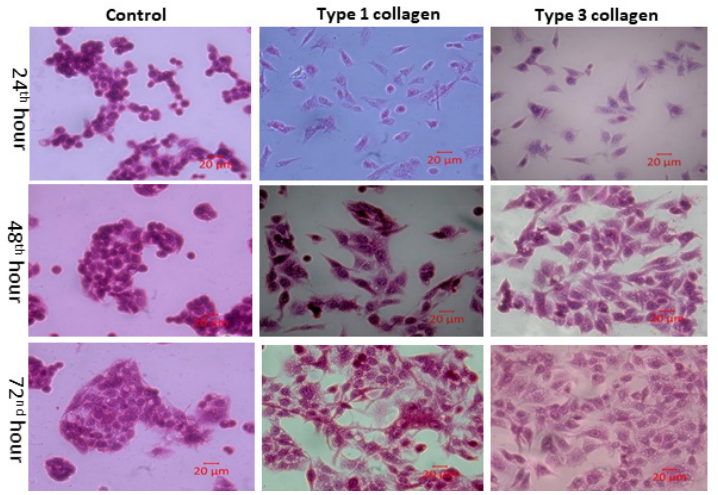

Figure 2: Cell morphological analysis using H&E staining. H&E: Haematoxylin-Eosin

Alpha fetoprotein (AFP)

It has been emphasised in various studies that AFP levels are important markers in liver cancer cells. In our project, we investigated the changes in the amount and localisation of this marker in HepG2 cells in the presence of Type 1 and Type 3 collagen under a confocal microscope (Ziess, LSM700, Germany) and performed quantitative data and statistical analysis. According to the results obtained; AFP expressed in HepG2 cells increased in media containing collagen during three different culture periods. At 24 h, the increase in AFP expression in HepG2 cells treated with Type 3 collagen medium was statistically significant compared with the control HepG2 cells ($p<0.05$). After 24 h of culture, there was an increase in AFP expression in cells treated with Type 1 and Type 3 collagen media compared with the control group, but no

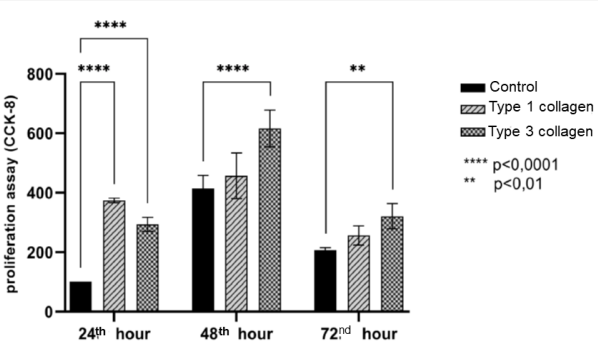

Figure 3: Proliferation analysis of HepG2 at different culture times

statistically significant difference was observed. At 72 h, there was an increase in AFP expression in HepG2 cells in culture medium supplemented with Type 1 collagen and a decrease in culture medium supplemented with Type 3 collagen, but no statistically significant difference was observed (Figure 4).

When we evaluated the confocal microscope images; specific binding with anti-AFP antibody was observed in HepG2 cells in all three culture media under a three-dimensional microscope. The AFP antibody was located in the cytoplasm close to the cell nucleus, indicating that collagen had no effect on the localisation of this antibody. Figures 5, 6, and 7 show confocal microscope images of HepG2 cells at three different culture stages in classical, Type 1 collagen, and Type 3 collagen culture media, respectively.

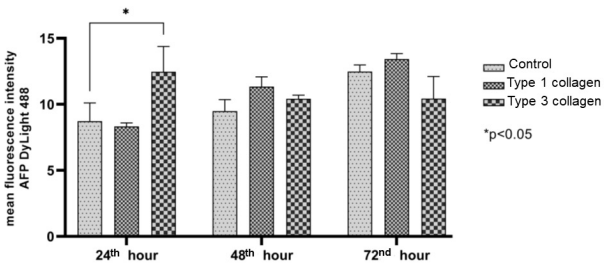

Figure 4: Bar graph showing the mean fluorescence intensity of AFP as mean ± SEM in HepG2 cells. AFP: Alpha fetoprotein

DISCUSSION

Hepatocellular carsinoma is most prominent risk factor is cirrhosis but in our country, the most common causes are viral hepatitis (Hep B and Hep C) and alcoholism (1-4). Nonalcoholic fatty liver disease, tobacco use, hemochromatosis, and diabetes are other risk factors. The incidence of COVID-19 increases with age and varies significantly depending on the geographic region. Early diagnosis of hepatocellular carcinoma significantly affects treatment and prognosis. Surgical resection and liver transplantation are the primary treatment approaches for early-stage hepatocellular carcinoma. If these methods are not available, chemotherapy is extremely useful in the advanced stage.

The microenvironment is extremely important for both normal and cancerous tissues. One of the most important components of the microenvironment is ECM components (8). The ECM consists of a non-cellular protein, glycoprotein, proteoglycan, and polysaccharide meshwork. The main component of the ECM is collagen. Collagens are often cross-linked and dispersed in such a way as to harden tissues. This elicits behavioural effects on

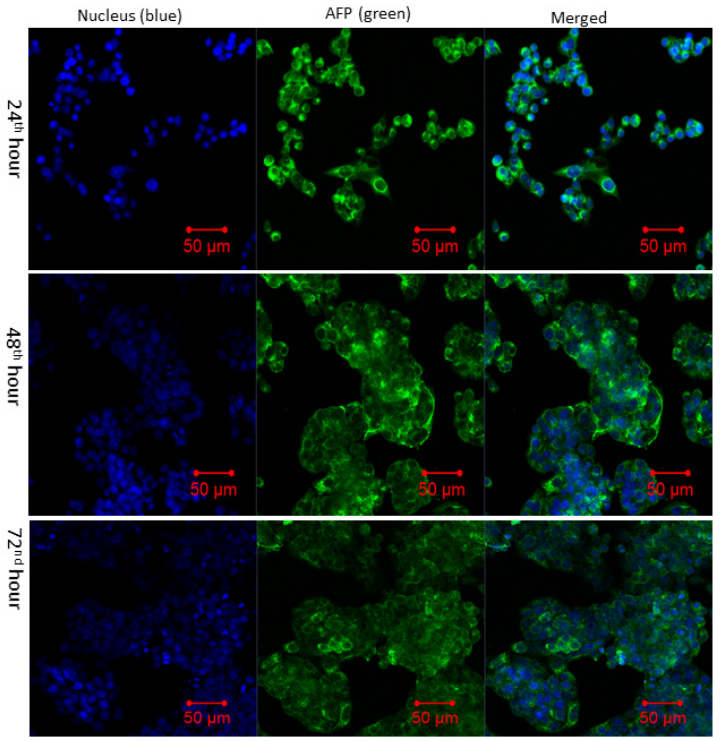

Figure 5: Confocal microscope images showing three levels of AFP expression in HepG2 cells cultured in conventional medium.

AFP: Alpha fetoprotein

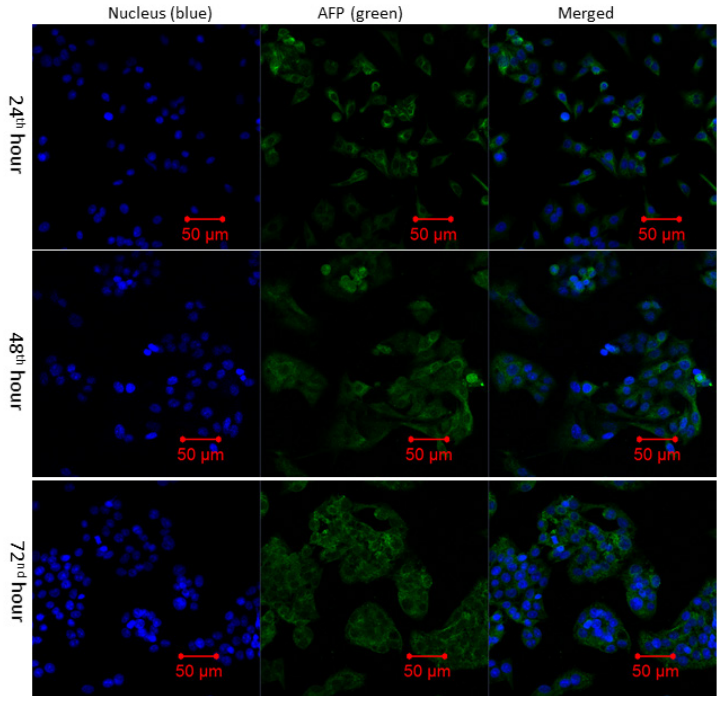

Figure 6: Confocal microscopy images of AFP expression in HepG2 cells in Type I collagen culture medium at three time points.

AFP: Alpha fetoprotein

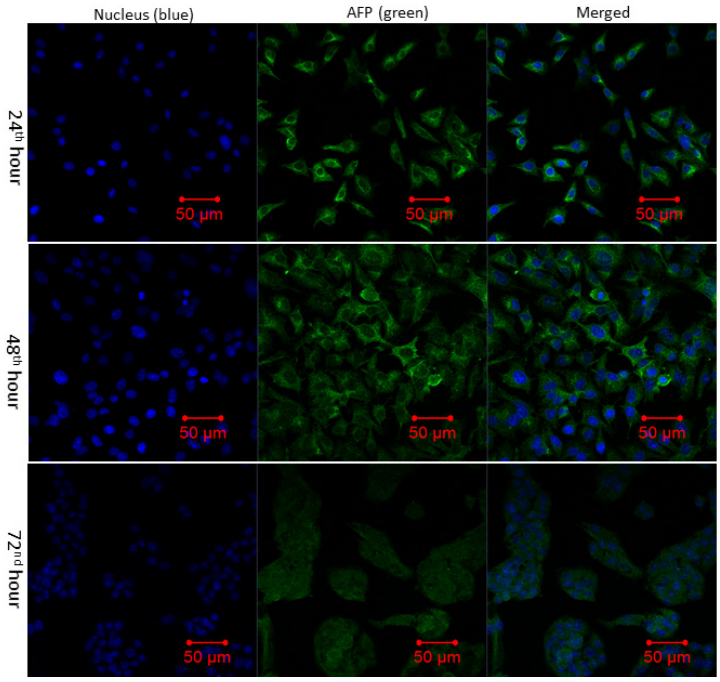

Figure 7: Confocal microscope images of AFP expression in HepG2 cells cultured in Type 3 collagen medium at three time points.

AFP: Alpha fetoprotein

surrounding tumour cells and regulates cell proliferation, differentiation, gene expression, migration, metastasis, and survival; thus, collagens directly influence the hallmarks of cancer (8, 9).

Molecular methods have shown that the level of Type 1 collagen is much higher in HCC samples than in normal liver samples (10). Type 3 collagen is the second most abundant collagen in the extracellular matrix. It is found primarily in the vascular systems, intestine, liver, skin, and lungs. Similar to Type 1 collagen, the distribution of Type 3 collagen is increased in many types of cancer (head and neck cancers, breast cancer, pancreatic cancer, colorectal cancer) (11-15).

Previous studies have shown that pancreatic cancer cells growing on Type 3 collagen exhibit increased proliferation, migration, and decreased expression of E-cadherin (16). Furthermore, Type 3 collagen plays a role in the invasion and metastasis of glioblastoma cells. These cells exhibit a high invasion and migration response when exposed to Type 3 collagen, and antibodies against Type 3 collagen inhibit these processes (17). Another study reported that Type 3 collagen altered some genes when invasive prostate cancer cells interacted with bone marrow stromal cells within the bone microenvironment. This interaction is important in demonstrating the involvement of Type 3 collagen in invasion and metastasis (18).

In a study comparing the cell spreading ability of human lung cancer cells on collagen Types 1 and 3 substrates with normal human tracheal epithelial cells; they showed that three different adenocarcinoma cell lines gradually started to contract after the initial spreading on Type 3 collagen and became round in 24 h. They suggested that their results showed that there may be a correlation between the degree of malignancy of human lung cancer cells and their ability to spread on collagen substrate and that cell spreading ability may be regulated by Type 3 collagen in some lung cancer cells (18). Wang et al. investigated Type 3 collagen expression and its roles in modulating lung carcinoma growth, viability, and apoptosis. They found that COL3A1 overexpression was associated with increased cell growth and clone formation but decreased cell apoptosis, whereas reduced COL3A1 expression led to decreased cell growth and clone formation and increased cell apoptosis (19).

In a study investigating how Type 1 collagen could restrict tumour expansion as a mechanical barrier, they found that Type 1 collagen expressed by cancer-associated fibroblasts (CAF) suppressed tumour growth by mechanically restraining tumour spread (20). In another study, they demonstrated that human breast cancer (BC) cells growing in culture media completely devoid of serum and seeded on Type 1 collagen coating exhibited a lower apoptotic rate and a decrease in Bax expression than those grown on plastic, indicating that Type 1 collagen promoted BC cell survival (21).

Collagen in the tumour microenvironment plays an important role in the regulation of tumour progression. Another study showed that Type 3 collagen, a component of tumour stroma, regulates myofibroblast differentiation and scar formation after cutaneous injury. In mouse and human breast cancer cell lines cultured at low concentrations of Type 3 and high concentrations of Type 3, it was shown that high concentrations were more effective in suppressing processes that are important in metastasis, such as surface adhesion and invasion. And it was stated that proliferation increased and cell death (apoptosis) decreased in mouse breast carcinoma cell lines in culture medium with low concentrations of Type 3 collagen. This mechanism has been mechanistically attributed to Type 3 collagen, which suppresses the procarcinogenic microenvironment by regulating stromal organisation, including the density and alignment of fibrillar collagen and myofibroblast (22).

CONCLUSION

It was determined that HepG2 cells cultured in both types of collagen have a morphologically similar structure, and Type 1 and Type 3 collagen have a proliferation-enhancing effect on cancer cells. AFP, an indicator of liver cancer, is found to be high in culture media containing Type 1 and Type 3 collagen, suggesting that there is a tendency towards poor prognosis for AFP expression of collagen types.

Ethics Committee Approval: Since the study is a cell culture study, ethics committee approval is not required.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- R.G.A., Z.A., H.M.; Data Acquisition- Z.A., B.E., G.D.; Data Analysis/Interpretation- Z.A., B.E., G.D.; Drafting Manuscript- B.E., Z.A.; Critical Revision of Manuscript – Z.A., R.G.A.; Final Approval and Accountability- Z.A., R.G.A.; Technical or Material Support- R.G.A.; Supervision- R.G.A.

Conflict of Interest: The authors have no conflict of interest to declare.

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