BioNanoScience (2018) 8:522–536
https://doi.org/10.1007/s12668-017-0494-z

Effect of Curcumin and Gliotoxin on Rat Liver Myofibroblast Culture

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Published online: 29 December 2017

Abstract
Since the 1990s, when it was demonstrated by Hammel and others that liver fibrosis is reversible, researchers and physicians actively search for new antifibrotic therapies. In recent years, knowledge of liver fibrosis pathophysiology has greatly advanced and new cellular and molecular mechanisms were described. The cells that determine extracellular matrix components distribution are myofibroblasts, but their origin is diverse. They can be activated hepatic stellate cells (HSCs), portal fibroblasts (PF), or circulating mesenchymal stem cells of the bone marrow. Among large number of substrates to inhibit activation, to inhibit proliferation of myofibroblasts, and to induce their apoptosis we, chose curcumin and gliotoxin. Primarily, in the current work, we optimized the explantation culture method for isolation of hepatic myofibroblasts and received two different cultures—myofibroblasts of HSC and PF origin. Exposition of 50 μM curcumin and 0.1 μM gliotoxin was the most optimal; we observed suppression of hepatic myofibroblast activation and inhibition of their proliferation. These results extend the current knowledge of the cells within the liver fibrogenic populations and prove inhibitory influence of biologically active substances (curcumin and gliotoxin) on portal myofibroblasts.

Keywords Hepatic stellate cells · Portal fibroblasts · Myofibroblasts · Curcumin · Gliotoxin · Apoptosis · Liver fibrosis

1 Introduction

Liver fibrosis is an extensive substitution of extracellular matrix (ECM) in response to chronic injury [1]. In comparison to acute liver injury, when hepatocytes and cholangiocytes retain their possibility to proliferate and replace damaged cells, in the case of chronic liver diseases, the resource of hepatic parenchymal cells to regenerate fails and then starts the changes in ECM deposition to replace the damaged region or to isolate it from other intact areas that manifests like liver fibrogenesis. The nature of injury agent determines the cells, which respond to damage, and diverse types of ECM distribution: in chronic viral hepatitis and chronic cholestatic disorders—around portal tracts, and in alcohol-induced liver disease—in pericentral and perisinusoidal areas [2]. In recent years, knowledge of liver fibrosis pathophysiology has greatly advanced and new cellular and molecular mechanisms were described.

Being resident mesenchymal cells of the liver, hepatic stellate cells (HSCs) and portal fibroblasts (PF) [3] are one of the first cells that activate, transdifferentiate into myofibroblasts, and acquire fibrogenic potential in response to liver injury [4–9].

In the normal liver, HSCs are located in the perisinusoidal space of Disse and being a quiescent cell population, they are the major storage of vitamin A [10]. Under chronic injury, HSCs activate and transdifferentiate into myofibroblast-like cells with contractile, proinflammatory, and fibrogenic properties [11, 12]. This could be followed by the changes of HSC phenotype: whereas quiescent HSCs express markers of adipocytes (PPAR-γ, SREBP-1c, and leptin), activated HSCs express myogenic markers (α-smooth muscle actin (α-SMA), c-myb, and myocyte enhancer factor-2) [4]. Due to their features, they had been thought for a long time as a main "causer" of liver fibrosis and cirrhosis [13]. Briefly, the damaged hepatocytes and stellate macrophages of the liver (Kuppfer cells) release reactive oxygen species and mediators that stimulate the migration of white blood inflammatory cells to the damaged region [14–16]. Inflammatory cells in their turn activate HSC that also produce chemokines to modulate

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the activity of lymphocytes [17]. Thus, appears a vicious pathological circle where every cell type stimulates each other [18]. Being activated, HSCs become a source of inflammatory mediators, transdifferentiate into myofibroblasts, acquire fibrogenic potential, and start to produce components of ECM [19]. Among ECM components synthesized by HSC are collagen fibers (I, III, and IV) and then fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans. At the same time, HSCs also produce matrix metalloproteinases (MMPs) [20] and their inhibitors (tissue inhibitors of matrix metalloproteinases, TIMPs), which regulate ECM distribution. Thus, ECM accumulation is a result of both increased synthesis and decreased degradation of ECM [21].

In the recent years, it was shown that not only HSC possess fibrogenic activity, also portal fibroblasts (PF) under influence of damaged epithelial cells can transdifferentiate into myofibroblasts and initiate collagen deposition around portal tracts [22]. Interestingly, these cells were first described in 1961 by Carruthers and colleagues, who studied the rat portal tracts after bile duct ligation [23, 24], but the cells were forgotten then and only in last years they became under great interest again. Nowadays, the term PF defines fibroblasts, localized in the portal area and not originated from HSC [25]. Normally, PF can be found around the portal vein and they maintain integrity of the portal tract [26]. PF regulate cholangiocyte proliferation, create peribiliary stem cells niche by deposition of elastin and other components of microfibrils; they also maintain stability of the portal tract structures under increased ductal pressure [25, 27]. New interesting angiogenic property of PF was described in 2015—portal myofibroblasts overexpress COL15A1, the α1 chains of collagen XV, normally present in trace amounts in portal and periportal areas, but whose significant quantities are detected in the case of hepatocellular carcinoma and pathological angiogenesis during liver fibrosis [28]. Due to the localization, PF react first to the chronic cholestatic liver diseases and biliary obstruction [29] and become the main source of connective tissue in the case of biliary cirrhosis [26, 30]. HSC will be also involved in fibrogenesis, but later with disease progression [27, 31, 32].

Along with these two types of myofibroblasts (activated HSC and PF), some authors distinguish a third one—myofibroblasts with intermediate phenotype and unknown origin, probably circulating mesenchymal stem cells of the bone marrow [33–35]. In some of the experimental works, it was shown the contribution of smooth muscle cells of vascular tunica media [36], second layer cells around the centrilobular veins [37], and capsular fibroblasts in the Glisson's capsule [38] or even epithelial-mesenchymal transition [39], but it still remains controversial.

As far as myofibroblasts could be of different origins, there is a question to distinguish them. The generally accepted main marker of myofibroblasts is the presence in their cytoplasm of α-SMA. To determine the origin of the myofibroblasts (HSC, PF, or bone marrow cells), we can turn to their antigenic properties. The resident HSC cells are characterized by expression of desmin and glial fibrillary acidic protein (GFAP)—markers, expressed even after HSC transdifferentiation and distinguishing HSC myofibroblasts from other types [19]. Generally, the regulation of cellular growth and function is under transcription factor control. Such a transcription factor in HSC is nuclear factor κB (NF-κB), whose increased levels and nuclear translocation are associated with HSC activation. [40–42]. HSC are also characterized by the absence of hematopoietic markers (CD45, CD34) and Thy-1 (glycophosphatidylinositol-associated glycoprotein on the outer cell membrane that has previously been detected in fibroblasts of certain organs, CD90). Myofibroblasts, resulting from epithelial-mesenchymal transformation, are characterized by the absence of CD45 expression and production of markers of hepatocytes (Albumin, Alb) and cholangiocytes (cytokeratin-19, CK-19) [39]. The cells from circulating bone marrow cells present CD45+/Coll1+ myofibroblasts. PF differ from HSC by the following features: they can express elastin and Thy-1, do not accumulate retinoids, and do not express desmin, CD146, or neural markers (GFAP) [43].

As far as it was shown that liver fibrosis is reversible [44], the search for the ways of myofibroblast fibrogenic potential regulation is under great interest [45]. However, to study the phenotype, features, and activation of the myofibroblasts, it is better to isolate these cells. Methods of rodent and human HSC isolation were developed in the 1980s [46, 47], which principle is based on collagenase-pronase liver perfusion with consequent separation of the vitamin A-containing HSC by density gradient centrifugation in Nycodenz [48]. For further in vitro activation of HSC and their transdifferentiation into myofibroblasts, myofibroblast was offered prolonged cultivation of HSCs on plastic culture plates [49].

Methods that would allow isolation of HSC, which do not contain vitamin A in cytoplasm, activated myofibroblasts, or PF, are not yet standardized. We assume that an alternative method to obtain activated cells, especially myofibroblasts, could be an explantation tissue culture method. Explantation tissue culture is a biological technique to organotypically culture cells from a piece of tissue or organ removed from a plant or animal [50]. The principle is that cells within the piece appear to be in stress, excrete various biological substances, which via paracrine, and autocrine ways will influence on activation of the tissue fragments cells, on their differentiation into myofibroblasts with further outgrowth onto the surface of culture dish. These primary cells can then be later expanded, cultivated, and transferred into fresh dishes. The major advantage of explantation culture is the maintenance of near in vivo environment for a short time [50]. For explantational outgrowth from liver are able only the most viable, activated cells that have the ability to migrate, contractile activity, express α-SMA—marker of myofibroblasts. Some authors describe different methods of PF isolation, based mainly on the enzymatic cleaning of the bile duct structures of the liver parenchyma

fragments [30]. We decided to check whether this method is applicable without enzymatic digestion of the tissue to decrease the enzymatic damage of the cells.

Many biochemical agents are used to stimulate HSC apoptosis in vitro and in vivo, among them are gliotoxin and curcumin [51].

Gliotoxin is a fungal metabolite of the epipolythiodioxopiperazine class of secondary metabolites. Due to its antibacterial, antifungal, and antiviral activities, gliotoxin is thought to be a promising therapeutic agent [51, 52]. It is known that gliotoxin induces apoptosis of thymocytes, peripheral lymphocytes, macrophages, P815 mastocytoma cells, fibroblasts line L929, and HSC in vitro, and also cells of the thymus, spleen, lymphatic nodules of mesentery, and liver in vivo [53]. Gliotoxin-induced apoptosis of human HSC culture was demonstrated in 2003 by Young-Oh Kweon and collegues [54]. However, the mechanism of apoptosis induction remains unclear. It is known to have diverse biological activities including proapoptotic effects in vivo and in vitro. Gliotoxin was shown to induce in the liver:

1) HSC apoptosis and reduced hepatic fibrosis in CCl_4 rat model [52].
2) Might induce adverse effect on other non-parenchymal cell types.
3) Demonstrates immunosuppressive effects on liver cells [53].

Similarly, curcumin (or (1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is also an important pharmacological agent. It is derived from tropical plant *Curcuma longa*, for thousands of years, and is being used as food spice and for medicinal purposes. Many signaling molecules are modulated by curcumin, such as molecules that activate inflammatory process, transcription factors, enzymes, protein kinases, reductases, carrier proteins, cell survival proteins, drug resistance proteins, adhesion molecules, growth factors, receptors, cell cycle regulatory proteins, chemokines, DNA, RNA, and metal ions [55].

The effect of curcumin on the process of fibrogenesis in the liver has been studied for a long time. Known for its effect on various cellular processes: adhesion, gene expression, and secretion of various growth factors and mediators [56], it participates in angiogenesis and immunomodulation [57]. In addition, curcumin shows properties of the inhibitor of oxidative stress and inflammation [58]. There were numerous studies of the influence of curcumin on cell cycle of various cells lines. [59]. It is still unknown why the equivalent concentrations of curcumin induce apoptosis of activated HSC and have no effect on quiescent HSC and other liver cells such as hepatocytes. Lin et al. studied the effect of curcumin on immortalized cell line HSC-T6 and showed that low concentrations of curcumin (1.25–10 μM) had no effect on cell survival, whereas a high concentration of it (20 μM) led to necrosis [60]. Curcumin provides antioxidative, anti-inflammatory, antifibrogenic, and antiproliferative effects on HSC. Interestingly, curcumin can induce apoptosis and prevent activation and proliferation of HSC; therewith, it prevents production of ECM [61–64].

Worth to note is the study of Baghdasaryan, who investigated the effect of curcumin on the PF and cholangiocytes of mice Mdr2−/− and showed that curcumin reduces proliferation and activation of portal fibroblasts via the ERK1/2 signal pathway [65]. In addition, the efficacy of curcumin in the treatment of liver fibrosis indicates the observed decrease of α-SMA and the collagen I synthesis in the liver [66]. Curcumin also leads to suppression of connective tissue growth factor (CTGF) gene expression in activated HSCs in vitro [67].

In recent years, another interesting aspects of the antifibrotic effect of curcumin on HSC were published: (a) via inhibition of hedgehog signaling in HSC, curcumin regulates metabolism and transdifferentiation of HSC into myofibroblasts [68]; (b) curcumin inhibits aerobic glycolysis associated with activation of adenosine monophosphate-activated protein kinase HSCs [69]; (c) curcumin promotes apoptosis of activated HSC by inhibiting the expression of cytokines related to the MyD88 pathway [70]; and d) curcumin can also induce senescence of HSC [71]. However, the basic effect of curcumin is based on inhibition of NF-kB-dependent genes expressions [72].

Regarding the role of portal fibroblasts in the synthesis of extracellular matrix and their great impact in liver fibrosis development, the study of curcumin and gliotoxin influence on HSC and PF is important to search for new strategies of liver fibrosis therapy. PF and HSC start to produce components of extracellular matrix after their activation. In most of the researches, it used quit high concentrations of curcumin and gliotoxin to induce apoptosis of myofibroblasts. We suppose that it would be better if the cells remained alive and just reduced their proliferation activity, because these cells also play an important role in liver regeneration. Thus, using optimal concentrations of curcumin and gliotoxin, it is possible to inhibit activation of the cells, return them to quiescent phase.

Within this study, we consider to isolate myofibroblasts of different origins by explantation culture from liver parenchyma and bile duct region and to study the effect of different concentrations of gliotoxin and curcumin on rat liver myofibtoblasts in vitro. The aim is to identify optimal concentrations of gliotoxin and curcumin to inhibit myofibroblasts.

2 Materials and Methods

The whole work was performed on newborn rats (E4, *Rattus norvegicus*, Wistar line, received from Laboratory Animal

Breeding Facility "Pushchino," 187 animals). All rats were handled according to local institutional animal care and use committee guidelines of Kazan Federal University on the use of laboratory animals (ethical approval by the Institutional Animal Care and Use Committee of Kazan State Medical University N9-2013). Animals were housed in an individually vented cage system with a 12 h light-dark cycle and received free access to standard chow and water.

2.1 Preparation and Culture of Hepatic Myofibroblasts

The hepatic myofibroblasts were isolated by explantation method. To optimize the technique, we isolated separately liver parenchyma tissue and region of bile ducts. For this, rats were sacrificed by decapitation and thereafter under guideline of binocular microscope (Stemi DV4 Carl Zeiss, Germany) and using sterile instruments, liver capsule was removed; then, parenchyma fragments and bile duct region (left, right and common hepatic ducts) were isolated. Inside the hood, the rest of parenchymal tissue from bile ducts was mechanically removed. Isolated bile duct regions and liver parenchyma fragments were separately placed in wells of 6-well plates and smaller 3-mm^3 fragments were placed inside the 24-well plates. Cells were grown in culture medium DMEM supplemented by 10% FBS, 200 mM L-Glutamine, and penicillin-streptomycin (PanEco) [73]. For better adhesion and immobility of the tissue inside the well, the culture medium covered only a half of the tissue fragment.

On the third day after seeding, the medium was changed and the tissue fragment (bile duct region and liver parenchyma fragments, respectively) was removed and embedded following a standard technique in paraffin or frozen in Neg-50™ Frozen Section Medium (ThermoScientific). Placed in Neg-50™, samples were immediately frozen at − 80 °C and was used to produce thin 5-μm slices on a cryostat microtome-NM560 Cryo-Star (Carl Zeiss, Germany).

Medium of the growing cells was changed every second day, and on the 3rd, 5th, 7th, and 12th days, cells from 6-well plate were collected for Western blot and flow cytometry analysis and the cells from 24-well plate were immunocytochemically stained. Part of the cells from 6-well plates after removal of the tissue fragment (3rd day) had been further cultivated next 7 days and seeded into 25-cm^2 plates at a standard growth medium. Upon reaching 80%, confluence of the monolayer cells was passaged.

2.2 Western Blot Analysis

In brief, at the end of the experimental procedure, proteins from cultured hepatic myofibroblasts were purified and protein content was determined by the BioRad protein assay (BioRad, Munich, Germany). After gel electrophoresis and semi-dry protein transfer, PVDF membranes were incubated in 10% dry milk solution for 30 min before immunodetection was performed using antibodies against α-SMA (mAb, 1:100), IkBα (rAb, 1:100), NF-kB (rAb, 1:500) or β-actin (mAb, 1:2000), respectively (Table 1). For detection of primary antibodies, blots were incubated with horseradish peroxidase-coupled anti-mouse or anti-rabbit IgG antibody (Sigma Aldrich) diluted 1:5000 at room temperature for 2 h, respectively. Blots were washed again and peroxidase activity was detected using chemiluminescent protein detection kit Amersham ™ ECL ™ Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences AB). For image acquisition and densitometric analysis, we used the ChemiDoc ™ XRS + System (Bio-Rad, Singapore).

2.3 Immunocytochemistry and Immunohistochemistry Analysis

Immunocytochemistry analysis was performed by microscopy using AxioOberver.Z1 (Carl Zeiss, Germany). On the 3rd, 5th, 7th, and 12th days, hepatic myofibroblasts cultured on glass cover slips (Ø 12 mm) were fixed with paraformaldehyde (4%, 5 min), washed thrice with TBS, and incubated in TBS containing 0.1% Triton X-100 for 10 min at room temperature. Thereafter, cells were incubated for 1 h at room temperature using primary antibodies listed in Table 1. Cells were washed extensively and proceeded by post primary block and polymer of system Novolink (Novocastra, UK) for all antibodies and streptavidin-biotin CSA system (Dako, Denmark) for C-kit antibody. Visualization of the staining was performed with a colorimetric substrate 3-Amino-9-ethylcarbazole (AEC, Sigma Aldrich, USA). Nuclei were counterstained with hematoxylin (Novocastra, UK) according to standard procedures.

In the case of immunohistochemistry, the steps of the staining procedure were the same, except two steps: first step was deparaffinization by consequent incubation of the slices with toluene and absolute ethanol and thereafter for retrieval of antigens, the slices were boiled in citrate buffer for 30 min.

2.4 Flow Cytometry Analysis

Primary culture of hepatic myofibroblast on the seventh day was collected for the study by flow cytometry. To detect intracellular antigens, the cells were fixed in a commercial reagent CellFix (Becton Dickenson) on the basis of formaldehyde for 30 min at 4 °C; membrane permeability was provided by incubation in 0.1% solution of Tween-20 in PBS for 10 min. For staining of membrane antigens, permeability step was skipped and the fixation of the cells was carried out after the staining procedure (postfixation). The results were analyzed on a flow cytometer Guava easyCyte 8HT (Millipore).

Table 1 List of antibodies

Name of antigen	Company, cat. no., dilution
Desmin—protein of intermediate filament of muscle cells cytoskeleton, marker of HSC	Santa Cruz (SC14026), 1:100, Dako (M076001), 1:30
α-SMA—α-smooth muscle actin, marker of myofibroblasts, and smooth muscle cells	Santa Cruz (SC130616), 1:100, Dako (M0851), 1:50
Cytokeratin-18 (CK-18)—protein of intermediate filament of epithelial cell cytoskeleton (hepatocytes, cholangiocytes)	Dako (M7010), 1:20
Cytokeratin-19 (CK-19)—protein of intermediate filament of epithelial cell cytoskeleton (cholangiocytes, hepatoblasts, oval cells)	Dako (M0888), 1:20
Thy-1 (CD90)—membrane surface protein, one of the stem cell markers	Abcam (ab225), ICC 1:200
C-kit (CD117)—stem cells growth factor receptor	Novocastra (T595), 1:400
NF-kBp65—universal transcription factor that controls expression of the immune response genes, apoptosis, and cell cycle	Santa Cruse (SC372), ICC 1:50, WB 1:500
IkBα—inhibitor protein of NF-kB	Santa Cruse (SC 371), ICC, WB 1:50
MMP-9—matrix metalloproteinase 9, one of the enzymes that remodel ECM	Abcam (ab7299), 1:50
MMP-3—matrix metalloproteinase 9, degrade collagen II, III, IV, IX и X types, proteoglycans, fibronectin and elastin, activate other MMPs, such as MMP-1, MMP-7 and MMP-9	Santa Cruse (SC 6839), 1:50
Caspase-7—enzyme that plays central role in cellular apoptosis	Santa Cruse (SC 6138), 1:50
Albumin—marker of differentiated hepatocytes	Santa Cruse (SC 50536), 1:50
α-Fetoprotein (α-FP)—one of the hepatoblast proteins	Santa Cruse (SC 8108), 1:50
CD45—marker of blood cells (leukocytes)	Santa Cruse (SC 25590), 1:50
β-actin—protein of cells cytoskeleton, provides structure and integrity of the cells	Sigma Aldrich (Mab-A2228), WB 1:2000

2.5 Curcumin and Gliotoxin Incubation/Processing and Detection of Apoptosis

On the basis of published data about the effects of different concentrations of curcumin and gliotoxin on HSC, our observations selected three concentrations of curcumin (100, 50, and 30 μM) and gliotoxin (0.25, 0.1, and 0.05 μM) for processing the cell culture of hepatic myofibroblasts [74].

Upon forming a monolayer, the hepatic myofibroblasts grown on 6-well plate were incubated for 24 h with curcumin or gliotoxin at chosen concentrations. Thereafter, the supernatant was collected, centrifuged for 5 min at 500×*g* to gather swimming cells. The remaining cells were trypsinized, collected, and centrifuged for 5 min at 500×*g*. The first and second cellular pellets were combined and washed twice with 1× Binding buffer and stained with Annexin-V FITC PI (Sigma APOF 20TST, USA) according to the manufacturer's instructions. After that, we calculated the percentage of cells in different states using the flow cytometer Guava easyCyte 8HT (Millipore).

2.6 Study of Cell Index

Technology of xCELLigence Real-Time Cell Analyzer (Roche, Germany) is based on the use of microelectronic sensors, integrated into the bottom of special culture plates. With a predetermined frequency, the impedance is measured between the sensor electrodes that gives us the information about the density and attachment of the cells, having dielectric properties. We programmed the frequency of measurements to every 15 min during 4 days. Before starting the experiment, it was added 100 μl of culture medium in each well of the special plate and set the background value (subtracting the influence of culture medium/media containing the test substance on the impedance value) of the device. Then, seeded 5000 cells per well. After 24 h, the curcumin and gliotoxin respectively were added in chosen concentrations.

3 Results and Discussion

Hepatic myofibroblasts were isolated from two different liver fragments—liver parenchyma and bile duct region; they appeared to be two different myofibroblast cultures.

Myofibroblasts from Liver Parenchyma The first explantational outgrowth of the cells from liver parenchyma fragments was observed on the third day after seeding (Fig. 1a). The morphology of these cells resembled the morphology of HSC—large cells with numerous processes (Fig. 1b). Myofibroblast nature was confirmed by staining with the antibody against their marker—α-SMA that was present in phenotype of all the cells. The expression of α-SMA gradually increased, indicating further constant transformation of the isolated cells into myofibroblasts (Fig. 1c)—cells capable of contraction and migration [75]. Performing the immunocytochemical staining on the 3rd, 5th, 7th, and 12th days of their growth, there was determined strong expression of the rat's HSC marker desmin (Fig. 1d) at all

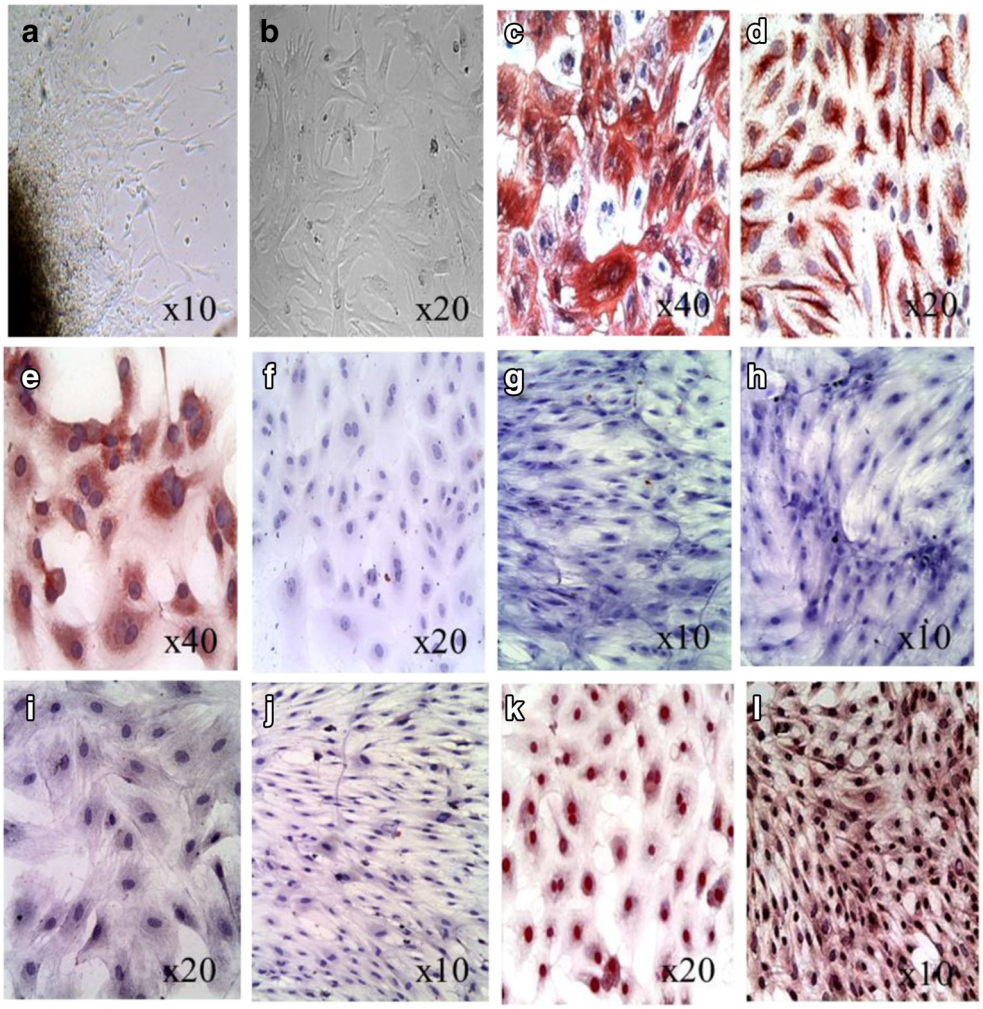

Fig. 1 Cell cultures of rat liver fragments: **a** during the cell isolation of the fragments liver, 3rd day; **b** monolayer myofibroblasts, 12-day culture; **c** α-SMA expression, 7 days; **d** desmin expression, 3 days; **e** C-kit expression, 7 days; **f** CK-18 expression, 7 days; **g** α-FP, × 10, 12 days; **h** albumin, × 10, 12 days; **i** CD45, × 20, 7 days; **j** MMP3, × 10, 12 days; **k** NF-kB, p65 isoform expression, 12 days; **l** IkBα expression, 7 days. Magnification: **a**, **g**, **h**, **j**, **l** × 10; **b**, **d**, **f**, **i**, **k** × 20; **c**, **e** × 40

stages of the experiment, which allows us to assume that the migrated cells were mostly activated HSC and only a few desmin-negative cells could be PF. Another marker expressed by every cell was C-kit—a marker of progenitor and stem cells (Fig. 1e), sustaining that these migrated cells had features of activated progenitor cells. To exclude the epithelial origin of the explanted cells, we checked expression of CK-18 (Fig. 1f) and CK-19 (data not shown). Expression of these antigens like expression of α-FP (Fig. 1g) and albumin (Fig. 1h) was not present that meant the absence of any epithelial cell admixture; there were neither hepatocytes nor cholangiocytes. To check the hematopoietic origin of the cells, we stained cell culture with antibodies against CD45 (Fig. 1i); this antigen was not expressed too, indicating an absence of blood cells in the experimental culture. Lack of expression of MMP-3 (Fig. 1j) and caspase-7 (data not shown) meant that the cells actively proliferated and there was no cell apoptosis.

It is worth to note that the high activity of the cells could be estimated by staining of cell culture with antibodies against NF-kB (Fig. 1k) and IkBα (Fig. 1l). We have observed a steady nuclear and cytoplasmic expression pattern in all cells and throughout the whole experiment. The expression of NF-kB mainly in the nucleus indicated a permanent activation of this transcription factor. This fact was confirmation of activated state of HSC, their transformation into myofibroblasts, and indirect confirmation of their fibrogenic features.

High and stable expression of NF-kB, IkBα, and α-SMA proteins was also confirmed by Western blot (Fig. 2).

To distinguish whether the explanted cells were HSC or PF, we stained the histological slices of the paraffin-embedded tissue on the third day after seeding with antibodies against desmin. We observed necrotic changes in the center of the fragment, while at the periphery of the tissue, it was noted a large number of desmin+ HSC (Fig. 3).

Regarding these results, we can make the following conclusions. This explantation method from liver parenchyma fragment allows obtaining culture of rat liver myofibroblasts. By analyzing the phenotype of these cells and histological slices of the liver fragments, we can propose that derived myofibroblasts are the result of desmin+ HSC transformation mostly, but not PF (desmin-). HSCs are relatively more active, viable, and able to explant in compare to PF.

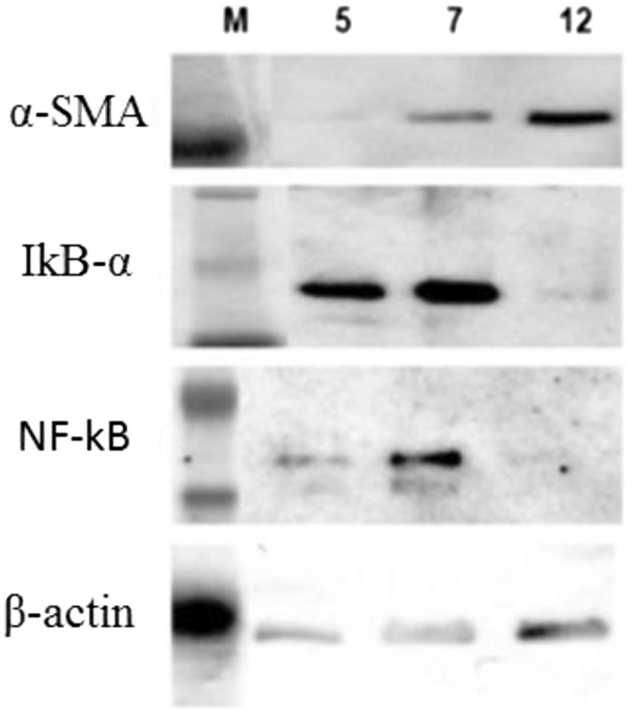

Fig. 2 Immunoblotting cell lysate proteins, obtained from the cell cultures on different stages of cultivation: M—molecular weight marker proteins; 5, 7, 12—duration of cell culture in vitro, days

The culture of myofibroblasts was characterized by high activity of the transcription factor NF-κB and its inhibitor IκBα that corresponds to high expression levels of these substances by activated HSC during liver fibrosis [76]. The observed in vitro activation process resembles the changes that occur with HSC and PF in vivo in the case of liver fibrosis. Thus, the model of explanted liver cells can be used in the preparation of liver myofibroblasts to conduct further experiments on inhibition of NF-κB and the study of the processes that lead to fibrosis of the liver. Understanding the origin of myofibroblasts and biology opens up new possibilities for finding necessary therapy.

Myofibroblasts from Bile Duct Region It is thought that portal fibroblasts, like HSC, in the case of chronic liver damage differentiate into myofibroblasts, expressing α-SMA. This could be modeled in vitro by cultivation of portal fibroblasts on cell culture dish or glass cover slips [77]. In our work, in vitro damage of the liver was mechanical damage during bile duct extraction. Under an operational microscope, we removed the surrounding liver parenchyma and placed in culture medium the bile duct regions that is together with portal vein and hepatic artery form the portal tracts.

The cellular explantational outgrowth was visualized on the third day of cultivation, similar to the previous group with explantational outgrowth of myofibroblasts from liver parenchyma. The cell population was heterogeneous: some of them had cuboidal, some—spindle-shaped morphology. To study

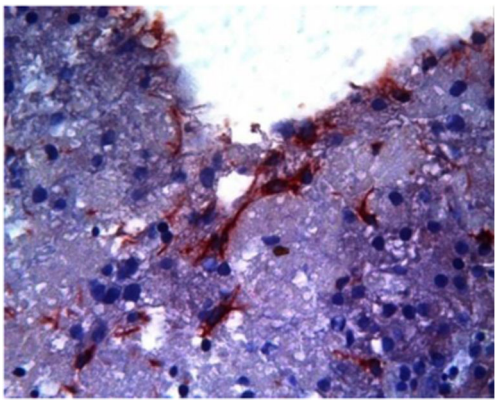

Fig. 3 Rat liver. Immunohistochemical reaction with antibodies to desmin, the third day of cultivation in vitro, × 40

the phenotype of the explanted cells, we performed flow cytometry and the results showed that there were only 7% of desmin+ cells, indicating that the bile ducts were properly purified from the liver parenchyma and there were a minimum of the HSC-originated myofibroblasts in the culture (Fig. 4b). This is also evidenced by immunocytochemistry for desmin in primary cell cultures and further in cultures of the first and second passages where only single desmin+ cells were determined (Fig. 5a–c).

Flow cytometry combined with Thy-1 staining showed that about 81% of the primary culture cells were Thy-1+ (Figs. 4c; 5d, e; 6b). In a number of studies, Thy-1 is described as a marker of bile duct cells and progenitor cells, while other researchers believe that the population of Thy-1+ is heterogeneous. There is also a report about the expression of Thy-1+ mesenchymal stem cell such markers as α-SMA and desmin [78]. Our study confirmed that PF were Thy-1+ cells, while hepatocytes, HSC, and liver macrophages did not express this marker. Thus, Thy-1 can be called one of the surface markers for the identification of the PF in vivo and in vitro [79].

At the 7th day of cultivation, 73% of the primary myofibroblast culture cells expressed the marker of myofibroblasts—α-SMA (Figs. 4a, 5f). Thereafter, the cells of the first and second passages did not express α-SMA, probably they transformed back into fibroblasts (Fig. 5g). Interestingly, by prolonged cultivation (passage 4), these cells resembled fibroblast morphology and phenotype, but later, some of the cells started to express α-SMA again (Fig. 6a), indicating repeated myofibroblast transformation of fibroblasts. These data confirm previously theorized possibility of fibroblasts to transdifferentiate into myofibroblasts [80]. The absence of epithelial markers (CK-18 and CK-19) confirmed that obtained cells were non-epithelial origin (Fig. 5h, i). Between these cells, there were no mature hepatocytes, they were albumin- and α-fetoprotein-negative. Thus, the cells, explanted from bile duct region fragments, were myofibroblasts and mostly had PF origin and only few of the cells were activated HSC.

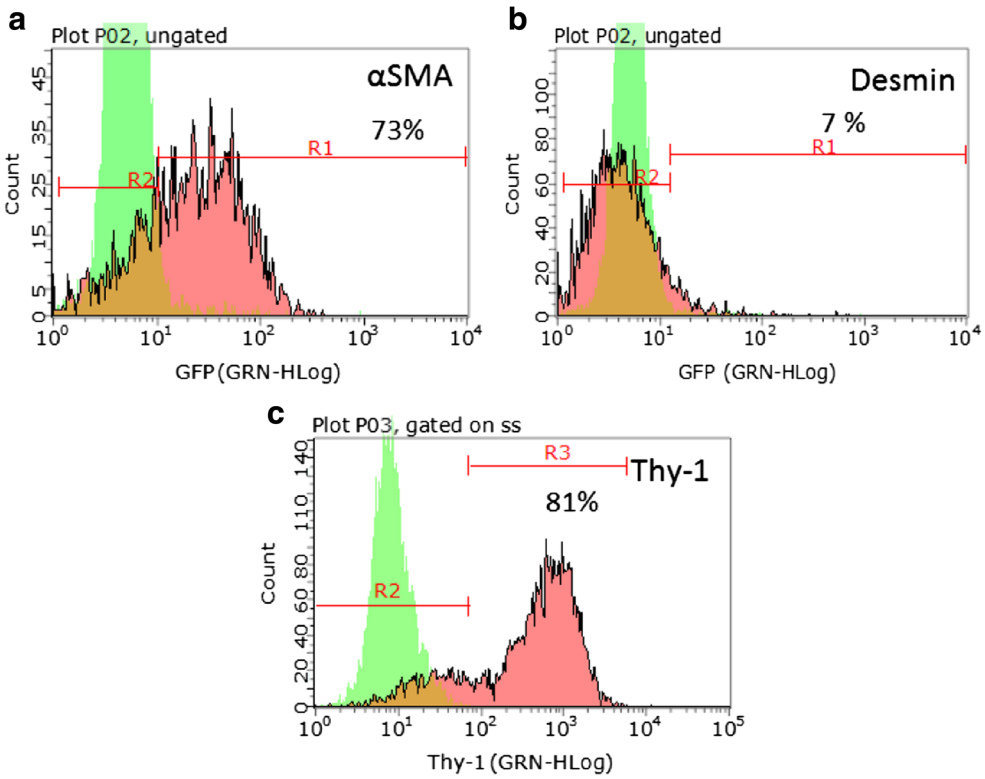

Fig. 4 Flow cytometry. Phenotype of primary culture cells isolated from the bile duct region: R1—cells expressing the marker; R2—cells not expressing the marker; R3—cells expressing Thy-1

Intermediate Conclusion In this model, we saw activation of fibroblasts, mostly PF, with further outgrowth from hepatic bile duct fragments. The presence of the 7% of desmin-positive myofibroblasts meant the presence of only 7% myofibroblast population, originating from HSC. According to the absence of epithelial markers (CK18, CK-19) and markers of mature

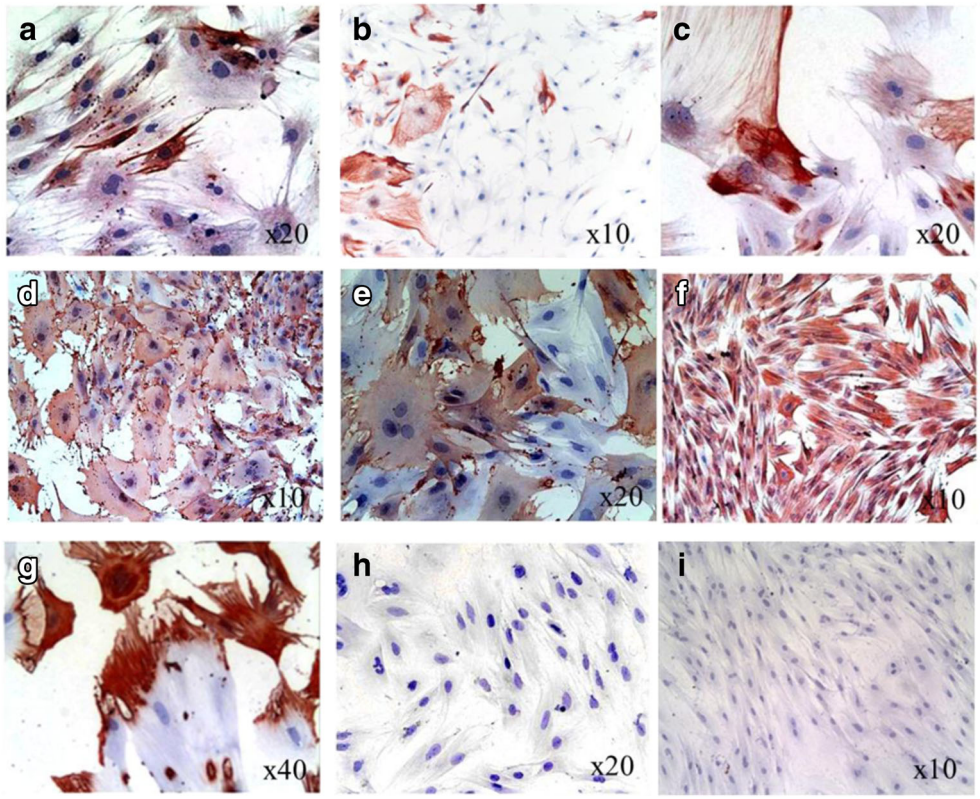

Fig. 5 Culture of cells derived from the bile duct region: **a** primary culture, the 7th day; **b**, **h**, **i** the culture of the first passage, the 5th day; **c** the culture of the second passage, the 5th day; **d**, **f** primary culture, the 12th day; **e**, **g** the culture of the second passage, the 7th day; **a**–**c** expression of desmin; **d**, **e** expression of Thy-1; **f**, **g** expression of α-SMA; **h** expression of CK-19; **i** expression of CK-18. Immunocytochemical reaction, the nuclei counterstained with hematoxylin. Magnification: **a**-**d**, **f**, **g**, **i**—× 10, **e**—× 20, **h**—× 40

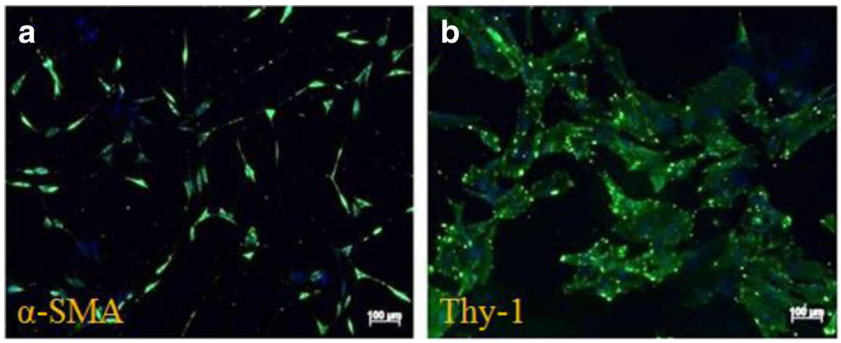

Fig. 6 The culture of cells isolated from the bile duct regions: **a** the culture of the 5th passage, the expression of α-SMA; **b** the culture of the 9th passage, expression of Thy-1. Nuclei visualization by DAPI staining (blue). Fluorescence microscopy

hepatic cells (albumin and α-FP), we suggest that there were no mature parenchymal cell contamination.

As a result, we can propose this tissue culture explantation model as a model for activated myofibroblast isolation. The region of the liver fragment allows to isolate myofibroblasts of different origins: from liver parenchyma fragment outgrow mostly myofibroblasts from activated HSC, from bile duct region—myofibroblasts from PF.

Other authors describe various methods for isolation of PF culture; their principle is based on enzymatic treatment of the bile duct structures from hepatic parenchyma [30]. Explantational method has several advantages in compare to these techniques: (1) simpler; (2) needs no additional substances, thus much cheaper; and (3) no enzymatic digestion of the tissue that damages the cells including PF. The disadvantage of explantational method is that this method does not allow to

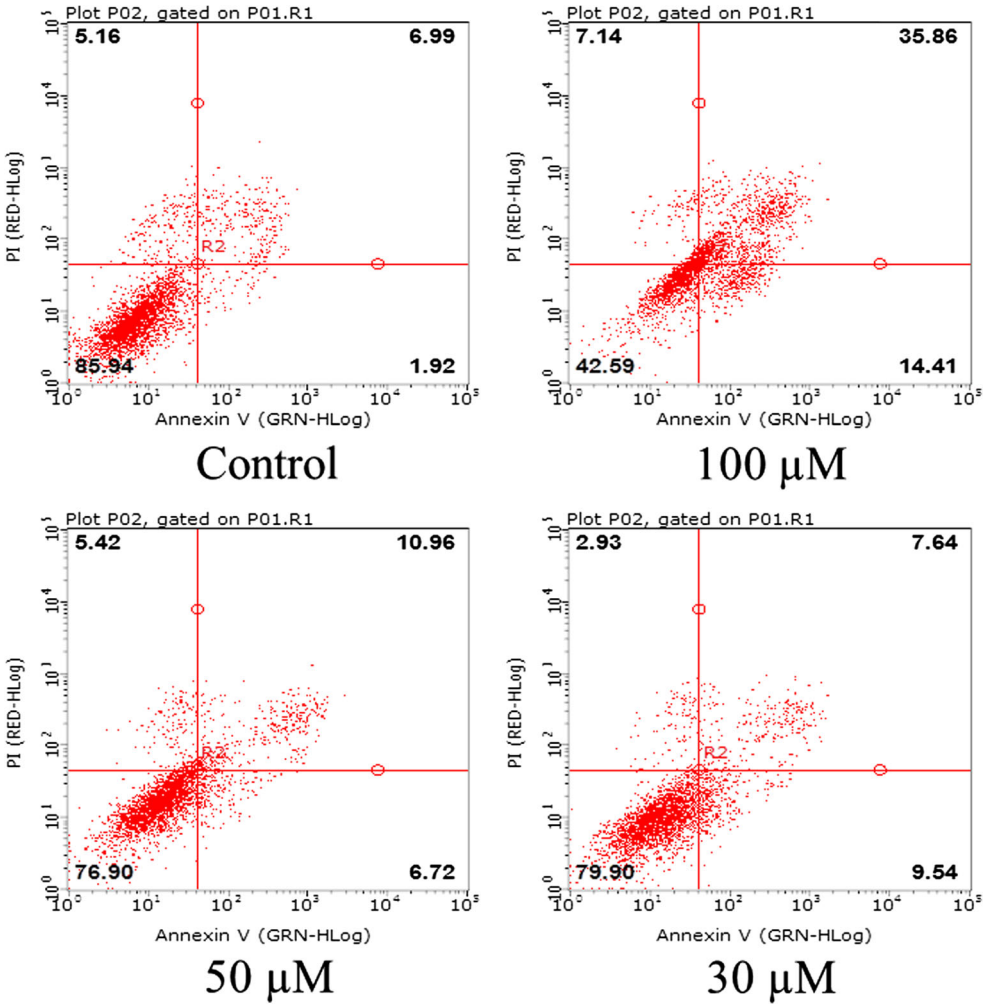

Fig. 7 The effect of curcumin on necrosis and apoptosis of portal myofibroblasts. Curcumin was added to the cell culture at concentrations of 100, 50, and 30 μM. After 24 h of incubation, the percentage of apoptosis and necrosis of the cells was determined by flow cytometry

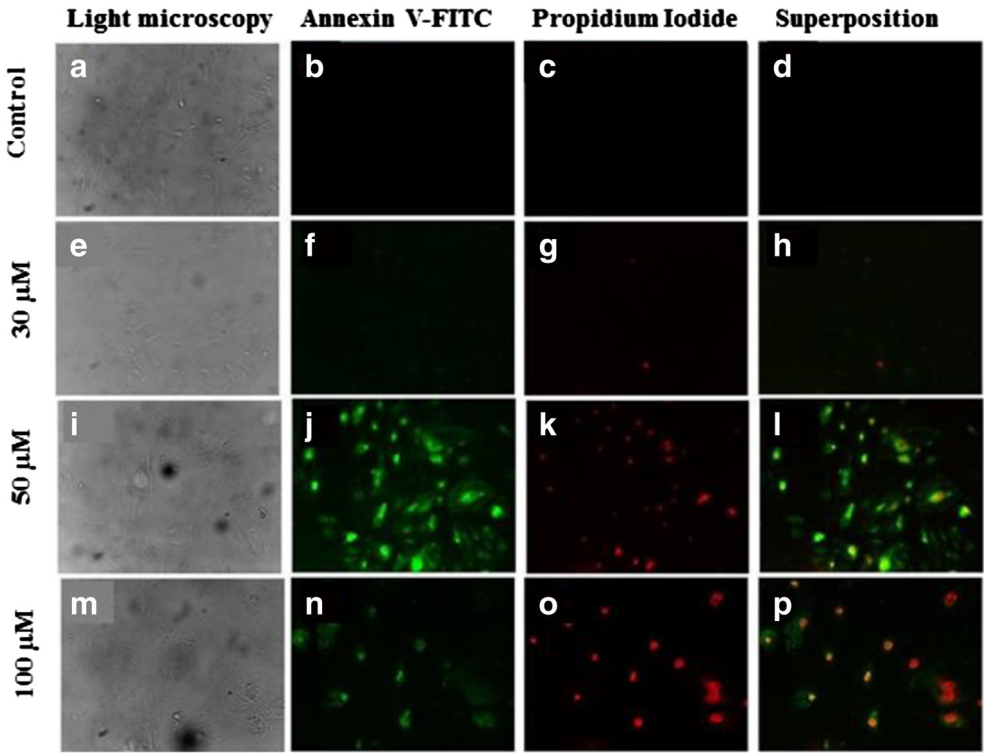

Fig. 8 Visualization of rat liver cells (which are in necrotic and apoptotic phases) incubated with various concentrations of curcumin (30, 50, 100 μM) using fluorescence microscopy (× 20). Cells in early apoptotic phase are stained with a green dye only (AnnexinV-FITC) and cells in necrotic phase are stained with green and red dye (Propidium Iodide). Living cells are not stained

isolate absolutely pure culture of portal myofibroblasts, but by liver fibrosis, there is activation of myofibroblasts from both origin (HSC and PF) and in this experiment, we can skip this disadvantage. To our mind, it is even better to study the effect of various substances on mixture of different myofibroblasts; it models the natural reaction of the liver cells.

In most of the clinical cases, liver fibrosis starts near the main portal tract or intrahepatic bile ducts with activation of myofibroblasts from PF, that is why we chose the second model to isolate the culture of PF by seeding the fragments of the bile duct region.

So far, one of the modern strategies of liver fibrosis treatment is prevention of myofibroblast activation; in our next experiments, we studied the influence of curcumin and gliotoxin on myofibroblast culture. Curcumin and gliotoxin are known to be inhibitors of myofibroblast activation and to induce apoptosis of myofibroblasts.

3.1 Analysis of the Effect of Curcumin on Rat Liver Portal Myofibroblast Culture in Various Concentrations

Performing the flow cytometry, we analyzed the quantity of the portal myofibroblasts that were in necrotic and apoptotic phase after incubation with curcumin. We got the following results (Fig. 7): at the highest concentration of curcumin (100 μM), cell necrosis count was greater (35.86%) than the cell apoptosis

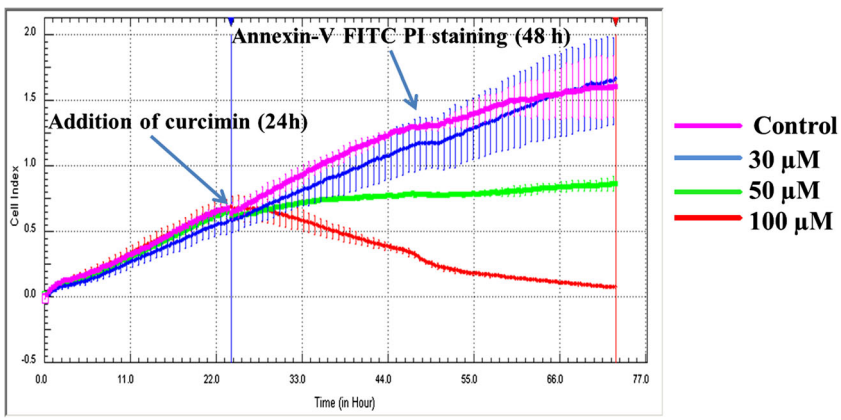

Fig. 9 The cell index (proliferation activity) of portal fibroblasts after curcumin exposure in various concentrations (30, 50, 100 μM). Microelectronic technology cell sensor device xCELLigance Real-Time Cell Analyzer

count (14.41%). At a lower concentration (50 μM), necrosis count was reduced to 10.96% and apoptosis count was two times reduced (6.72%). At the lowest curcumin concentration (30 μM), we observed the lowest necrosis count (7.64%), but interestingly, the apoptosis count increased (9.54%) and was higher than at 30 μM curcumin concentration.

The same results were obtained by immunocytochemical staining and study of the cell index. The highest concentrations of curcumin (100 μM) had the most toxic effect on cells. After, an initial period of adaptation of the cells to the substance began their apoptosis (Annexin-V+/PI-), and then, the cells were in late apoptosis/necrosis stage (Annexin-V+/PI+), which is reflected on the fluorescent photographs (Fig. 8m–p) and in descending curve of cell index showing a reduction of cell proliferation (Fig. 9). To the medium (50 μM) concentrations of curcumin cells adapted well, signs of early apoptosis were observed (Annexin-V+/PI-); only a few cells were in the stage of late apoptosis/necrosis (Annexin-V+/PI+) (Fig. 8i–l). Then, the cell index curve appeared on the plateau phase; however, these values were below the control numbers (no added compounds) (Fig. 9). This concentration can be considered as the most effective, as the cells retain the ability to proliferate, although at a lower level. It is seen that the growth of cells relative to cells in the control group decreased by about 50%. When exposed to low concentrations of curcumin (30 μM), only a few cells underwent apoptosis (Fig. 8e–h) and cell index was almost the same without changes (Fig. 9). It is known that curcumin blocks the expression of a broad spectrum of genes (PPAR-γ, α1-collagen, α-SMA, CTGF, receptor of TGF-β, PDGF, EGF), which indicates that curcumin acts on many signaling pathways, including, perhaps, the activation pathway of inflammation and fibrogenesis [81].

The study of the curcumin effect on cell growth of various cell lines in vitro indicates that curcumin has an effect on cells in a wide range of micro- and millimolar concentrations. As a result of its action, there is induced apoptosis and decreased generation of reactive oxygen species [59]. It is known that curcumin has a strong antioxidant effect, even stronger than vitamins C and E [82–85]. Since the lowest concentration of 30 μM curcumin showed almost no effect on the cell index, this concentration may be offered for use in vivo as an antioxidant. Depending on the concentration, curcumin is capable to induce apoptosis and necrosis of PF in vitro. We assume that the highest allowable concentration of curcumin that can

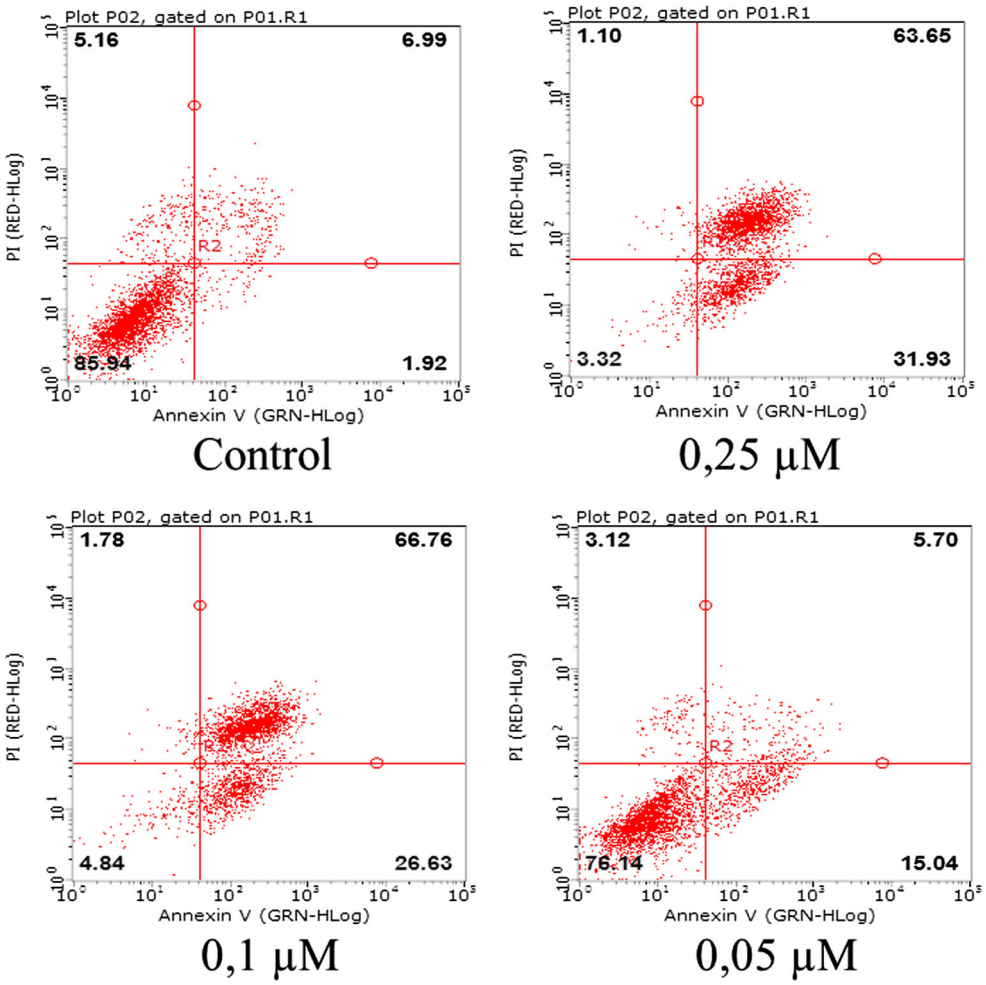

Fig. 10 The number of PF with apoptosis and necrosis after incubation with gliotoxin in various concentrations (0.05, 0.1, 0.25 μM). AnnexinV-FITC—a marker of apoptosis; PI—a marker of necrosis. Flow cytometry

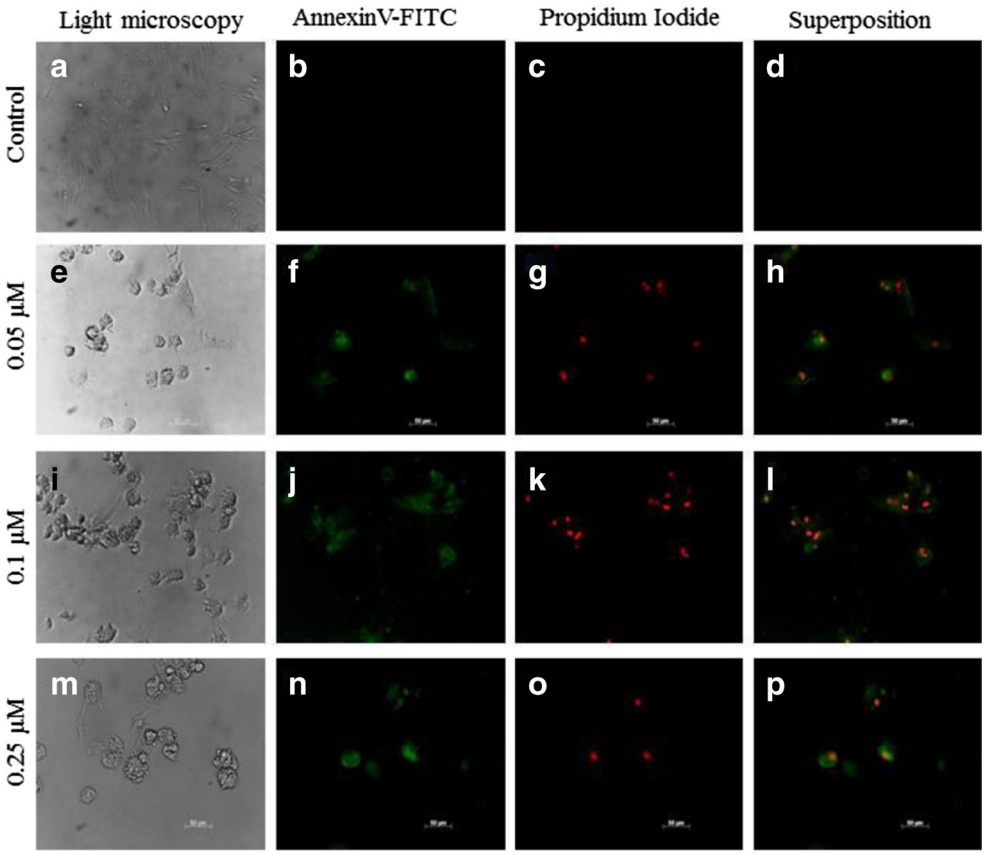

Fig. 11 Visualization of rat PF (which are in necrotic and apoptotic phases) incubated with various concentrations of gliotoxin (0.05, 0.1, 0.25 μM) using fluorescence microscopy (× 20). Cells in early apoptotic phase are stained with a green dye only (AnnexinV-FITC) and cells in necrotic phase are stained with green and red dye (Propidium Iodide). Living cells are not stained

be used to suppress activated PF and will have antifibrotic effect in vivo is 50 μM. However, the mechanism of its hepatoprotective action in vivo is not yet fully disclosed [86].

3.2 Analysis of the Effect of Gliotoxin on Rat Liver Cell Cultures in Various Concentrations

Incubation of PF culture with gliotoxin leads to changes of cell morphology—they became rounded, lost intercellular contacts, detached from the culture of plastic, and swam in the suspension; some of them exposed to necrosis (Figs. 10 and 11). These gliotoxin effects were due to the fact that gliotoxin inhibits the expression of surface adhesion markers, such as VCAM, ICAM, and E-selectin, and is involved in the reorganization of the cytoskeleton (e.g., actin) [87].

In the first 24 h, 0.05 μM gliotoxin had no effect on the cells. Cells were subjected for a short period of time to apoptosis (Figs. 10 and 11), but then recovered and after 72 h, their growth cell index was even higher than the one of control culture cells (Fig. 12). Incubation of PF culture with 0.1 μM gliotoxin induced apoptosis of PF and partially their necrosis (Figs. 10 and 11) and further increase of cell culture growth index was not observed (Fig. 12). Thus, gliotoxin in concentration of 0.1 μM inhibits the PF proliferation (Fig. 12).

The concentration of 0.25 μM was too toxic and causes necrosis of PF (Fig. 10, 11, and 12). In compare to curcumin,

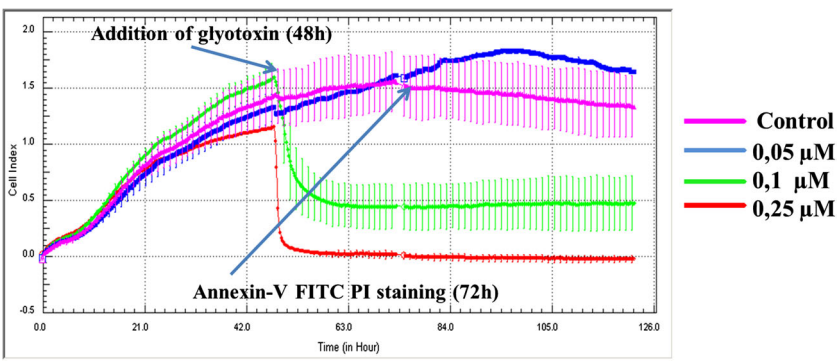

Fig. 12 The cell index (proliferation activity) of portal fibroblasts after addition of gliotoxin in various concentrations (0.05, 0.1. 0.25 μM). Microelectronic technology cell sensor device xCELLigance Real-Time Cell Analyzer

gliotoxin is more toxic for PF. Thus, by incubation of PF with gliotoxin in concentration of 0.25 μM, 63.65% of the cells died and 31.95% were apoptotic, alive remained only 3.32% of the cells (Fig. 10). With decrease of gliotoxin concentration (0.1 μM), the percentage of the dead and apoptotic cells decreased (49.86 and 24.90%, respectively), and the number of living cells increased—22.66% (Fig. 10). The smallest of the selected concentrations of gliotoxin (0.05 μM) did not lead to immediate cell death (necrosis 8.82%), but 15.04% of the cells were in apoptotic phase, which is almost eight times higher than the spontaneous apoptosis in the control cell culture (1.92%). The number of alive cells was determined to be 76.14% (Fig. 10). Effects of gliotoxin on cultured PF were also examined by inverted fluorescence microscopy after staining with AnnexinV-FITC and PI (Fig. 11).

Based on our results, the 0.1 μM concentration of gliotoxin is not toxic for the cells, but still it reduces the proliferative activity of PF and can have a significant effect on myofibroblast culture.

4 Conclusion

Explantational cell growth is an alternative method for myofibroblast isolation. It is important to note that this method allows to isolate myofibroblasts of different hepatic origins: from liver parenchyma outgrow myofibroblasts from activated HSC, from bile duct region fragments—myofibroblasts from PF. To study the ways of prevention of myofibroblast activation and induction of their apoptosis, there were two substances chosen—curcumin and gliotoxin. The effect of these substances on myofibroblast apoptosis has been shown to be concentration-dependent. Based on abovementioned results, the optimal concentration of curcumin is 50 μM and concentration of gliotoxin—0.1 μM. At these concentrations, we observed suppression of hepatic myofibroblast activation and inhibition of their proliferation, that means, that even after their treatment with gliotoxin and curcumin, cells will be able to participate in liver regeneration. The results extend the current knowledge of the myofibroblasts within the liver and open up new possibilities for biologically active substances (curcumin and gliotoxin) to influence on their activity and fibrogenic potential.

Funding Information The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University and subsidy allocated to Kazan Federal University for the state assignment in the sphere of scientific activities. Some of the experiments were conducted using the equipment of Interdisciplinary center for collective use of Kazan Federal University supported by the Ministry of Education of Russia (ID RFMEFI59414X0003), Interdisciplinary Center for Analytical Microscopy and Pharmaceutical Research and Education Center, Kazan (Volga Region) Federal University, Kazan, Russia.

Compliance with Ethical Standards All rats were handled according to local institutional animal care and use committee guidelines of Kazan Federal University on the use of laboratory animals (ethical approval by the Institutional Animal Care and Use Committee of Kazan State Medical University N9-2013).

Conflict of Interest The authors declare that they have no conflicts of interest.

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