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Activated stellate (Ito) cells possess voltage-activated calcium current

Hirosumi Oide [a], Michihiro Tateyama [b], Xian-En Wang [a], Miyoko Hirose [a], Tomoko Itatsu [a], Sumio Watanabe [a], Rikuo Ochi [b], Nobuhiro Sato [a,*]

[a] *Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[b] *Department of Physiology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*

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Abstract

We previously reported stellate (Ito) cells possess voltage-activated Ca^{2+} current. The activation of stellate cells has been indicated to contribute to liver fibrosis and the regulation of hepatic hemodynamics. The aim of this study was to investigate the relationship between voltage-activated Ca^{2+} current and activation of stellate cells. Voltage-activated Ca^{2+} current in stellate cells isolated from rats were studied using whole-cell patch clamp technique. L-type voltage-activated Ca^{2+} current was hardly detected in stellate cells cultured for less than 9 days. Ca^{2+} current was detected 12.5 and 69% of cells at the 10th and 14th day of culture, respectively. BrdU incorporation indicated cell proliferation was recognized over 50% of cells at the 3rd and 5th day of culture, respectively, then decreased significantly in a time-dependent manner. On the other hand, the expression of α-smooth muscle actin indicated cell activation increased from 7th day of culture and collagen type I mRNA appeared remarkably in cells cultured for more than 10 days. In this study, we concluded L-type voltage-activated Ca^{2+} current was recognized in activated stellate (myofibroblast-like) cells. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Stellate cell; Calcium current; Myofibroblast; α-Smooth muscle actin; Collagen type

1. Introduction

Hepatic stellate (Ito) cells have been shown to store vitamin A and produce extracellular matrix contributing to liver fibrosis [1,2]. On the other hand, they contain desmin [3] as intermediate filaments like muscle cells and have contractile proteins, such as actin and myosin [4]. These cells surround endothelial cells in the space of Disse. Hence, the regulatory role of stellate cells in hepatic microcirculation has been suggested in studies using cultured stellate cells by their contraction induced by vasoconstrictors such as endothelins and eicosanoids accompanied with an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and by their relaxation with nitric oxide [5,6]. Administration of endothelin-1 or alcohol elevated portal vein pressure in perfused liver [7,8]. Oxygen tension was reduced in the early stage of alcoholic liver injury and hepatic sinusoids contracted with endothelin-1 in rats treated chronically with ethanol [9,10]. Therefore, stellate cells might be responsible for changes in hepatic microcirculation in the pathogenesis of liver injury. Most of contractile cells, such as skeletal, cardiac and smooth muscle

Abbreviations: EGTA, ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HBSS, Hanks' balanced salt solution; Tris, Tris (hydroxymethyl) aminomethane; PMSF, phenylmethylsulfonyl fluoride; Na-DCA, Na-deoxycholic acid

* Corresponding author. Fax: +81 (3) 3813-8862;
E-mail: nsato@med.juntendo.ac.jp

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cells, have L-type voltage-activated Ca^{2+} channels [11,12]. We have recently demonstrated increases in $[Ca^{2+}]_i$ with high K^+ solution using fura-2 fluorometry and suppression of $[Ca^{2+}]_i$ with a Ca^{2+} channel blocker [13], evidence indicating the existence of voltage-activated Ca^{2+} channels in hepatic stellate cells. On the other hand, a transformation of quiescent cells to activated phenotype has been considered to be an important factor for the progression of liver injury [2]. Furthermore, the activation of stellate cells was reported to be associated with the development of contractility [14]. The present study was undertaken for the first time to evaluate biological characterization of the stellate cells containing voltage-activated Ca^{2+} current by electrophysiological technique, Western blot analysis of α-smooth muscle actin, BrdU staining and RT-PCR of collagen type I mRNA.

2. Materials and methods

2.1. Isolation and culture of stellate cells

All animals received humane care in compliance with institutional guidelines. Sprague-Dawley female rats (200–250 g) were anesthetized with pentobarbital sodium (5 mg/100 g body wt, i.p.). Hepatic stellate cells were isolated using collagenase digestion methods and a triple-layer density cushion of metrizamide as reported previously [13]. Briefly, The liver was perfused through the portal vein with Ca^{2+}, Mg^{2+}-free Hanks' balance salt solution (HBSS) at 37°C for 5 min at a flow rate of 26 ml/min. Subsequently, the liver was perfused with 0.05% collagenase (type I, Sigma, St. Louis, MO) at 37°C for 10 min at a flow rate of 20 ml/min. After the liver was digested, it was excised and cut into small pieces in 0.05% collagenase. The suspension was filtered through nylon gauze and the filtrate was centrifuged at $450\times g$ for 10 min at 4°C. Cell pellets were resuspended in buffer, parenchymal cells were removed by means of centrifugation at $50\times g$ for 3 min and the nonparenchymal cell fraction was washed twice with buffer. Cells were centrifuged on a triple-layer density cushion of Metrizamide (7, 13 and 18%; Sigma) at $1000\times g$ for 20 min at 20°C. The stellate cell fraction was collected from the upper layer. Desmin staining as a specific character for stellate cells [3] was positive over 90% of the cells. The yield of stellate cells using this method was $5–10\times10^5$/liver. Viability of the cells, evaluated by the trypan blue exclusion test, was more than 80%. 5×10^4 cells were seeded onto small pieces of glass coverslips and cultured in DMEM (Gibco Laboratories Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin sulfate) at 37°C with 5% CO_2. The culture medium was changed 24 h after cell preparation, then changed once after 3–4 days.

2.2. Patch clamp methods

Stellate cells were washed three times with 2 ml HBSS (37°C) and left at room temperature for longer than 1 h before they were used for patch clamp experiments. Glass coverslips bearing stellate cells were transferred to a circular chamber which had a shallow round central pool with a depth of less than 1 mm and a diameter of 10 mm. The chamber was mounted on the mechanical stage of an inverted microscope (Diaphoto, Nikon, Tokyo). The superfusate flowed down to the central pool and was drained out through a U tube, both by means of gravity. The main superfusate was modified Tyrode solution containing (in mM) NaCl, 135; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES, 5; and glucose, 10; and the pH was adjusted to 7.4 with NaOH. The macroscopic currents of stellate cells were recorded using the whole-cell patch clamp technique [15]. Patch pipettes were made of soft glass capillary tubes and had a resistance of 2–5 MΩ when filled with pipette solutions. The pipette solution for whole-cell recordings contained (in mM) *N*-methyl-D-glucamine (NMDG) Cl, 140; EGTA, 1; ATP, 5; and the pH was adjusted to 7.4 with Tris. The reference electrode contained 3 M KCl. The junction potential at the tip of whole-cell pipettes was −3 mV pipette positive and was not corrected in the evaluation of the membrane potential. Membrane capacitance was estimated from the capacity current elicited by step voltage pulses. The membrane potential of the patch membrane was corrected for this potential as in previous study [16]. EPC-7 patch clamp amplifier (List-Medical, Darmstadt) was used, and pClamp software (Axion, Foster

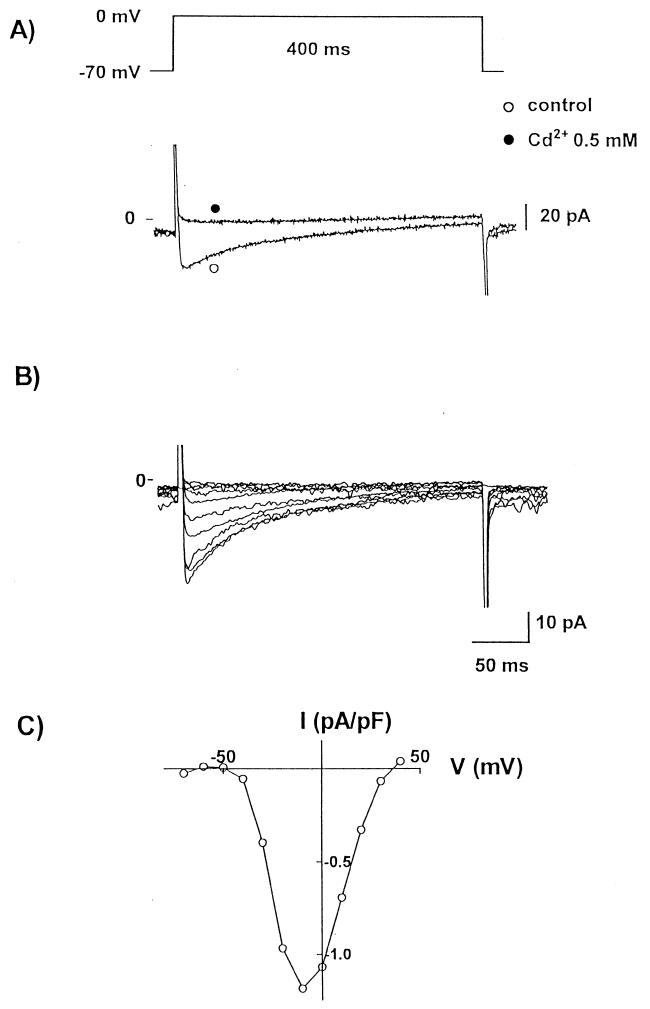

Fig. 1. Representative Ca^{2+} currents at each depolarizing pulses. (A) Superposition of current traces before (○) and after (●) administration of 0.5 mM Cd^{2+} for 400 ms to 0 mV from the holding potential of −70 mV. (B) Cd^{2+}-sensitive inward currents obtained by subtracting the currents in the presence of 0.5 mM Cd^{2+} from the control currents. They were elicited by a 400-ms step depolarization in 10-mV increments from a holding potential of −70 to +20 mV. (C) A typical relationship between peak amplitude of Cd^{2+}-sensitive inward currents and applied voltage. Vertical axis represents current density (pA/pF). Horizontal axis is the membrane potential (mV).

City, CA) was employed for both command-pulse delivery and data analysis.

2.3. Detection of cell proliferation

Proliferating cells were detected using 5-bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) labeling with incubation of the cells at 37°C with 5% CO_2 for 1 h at a concentration of 10 μg/ml BrdU followed by indirect immunostaining using a monoclonal antibody against BrdU [17].

2.4. Immunocytochemistry

To identify desmin, anti-desmin polyclonal antibody and rhodamine-conjugated swine anti-rabbit polyclonal antibody were used (Dako, CA).

2.5. Western blot analysis

To evaluate the expression of α-smooth muscle actin during culture, cells were lysed with RIPA buffer containing Tris-Cl, 50 mM; NaCl, 150 mM; EDTA, 10 mM; NaF, 20 mM; PMSF, 0.25 mM; Na-DCA, 1%; Triton X-100, 1% and leupeptin, 0.5 μg/ml, at the 5th, 7th, 10th and 14th day of culture. The protein concentration of the lysates was estimated using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples equivalent to 5 μg were electro-phonetically separated on a 5∼10% gradient SDS-PAGE gel and transferred onto Immobilon-P transfer membrane (Millipore, Bedford, MA). Immunoblots were blocked with 2% skimmed milk/Tris-Cl overnight at 4°C, and then incubated with anti-α-smooth muscle actin antibody (American Research Products, Belmont, MA) for 1.5 h. The bands were visualized using the enhanced Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and DAB substrate kit (Dako, Carpinteria, CA).

2.6. RT-PCR for collagen type I mRNA

Total RNA was extracted from stellate cells at the 5th, 7th, 10th and 14th day of culture using Trizol Reagent (Gibco BRL). 1 μg total RNA was transcribed at 42°C for 55 min in 22 μl of reverse transcriptase buffer containing 0.2 μg Oligo (dT) primer (Invitrogen, Carlsbad, CA) and 200 U of Moloney murine Leukemia virus reverse transcriptase (Gibco BRL). 1 μl of RT reaction was used as first strand cDNA for PCR. PCR was performed with rat collagen I (α1), sense 5′-ACAAgggTgAgACAggCgAA-3′ and antisense 5′-ggACCAAACAggAACCACTA-3′, for 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and primer extension

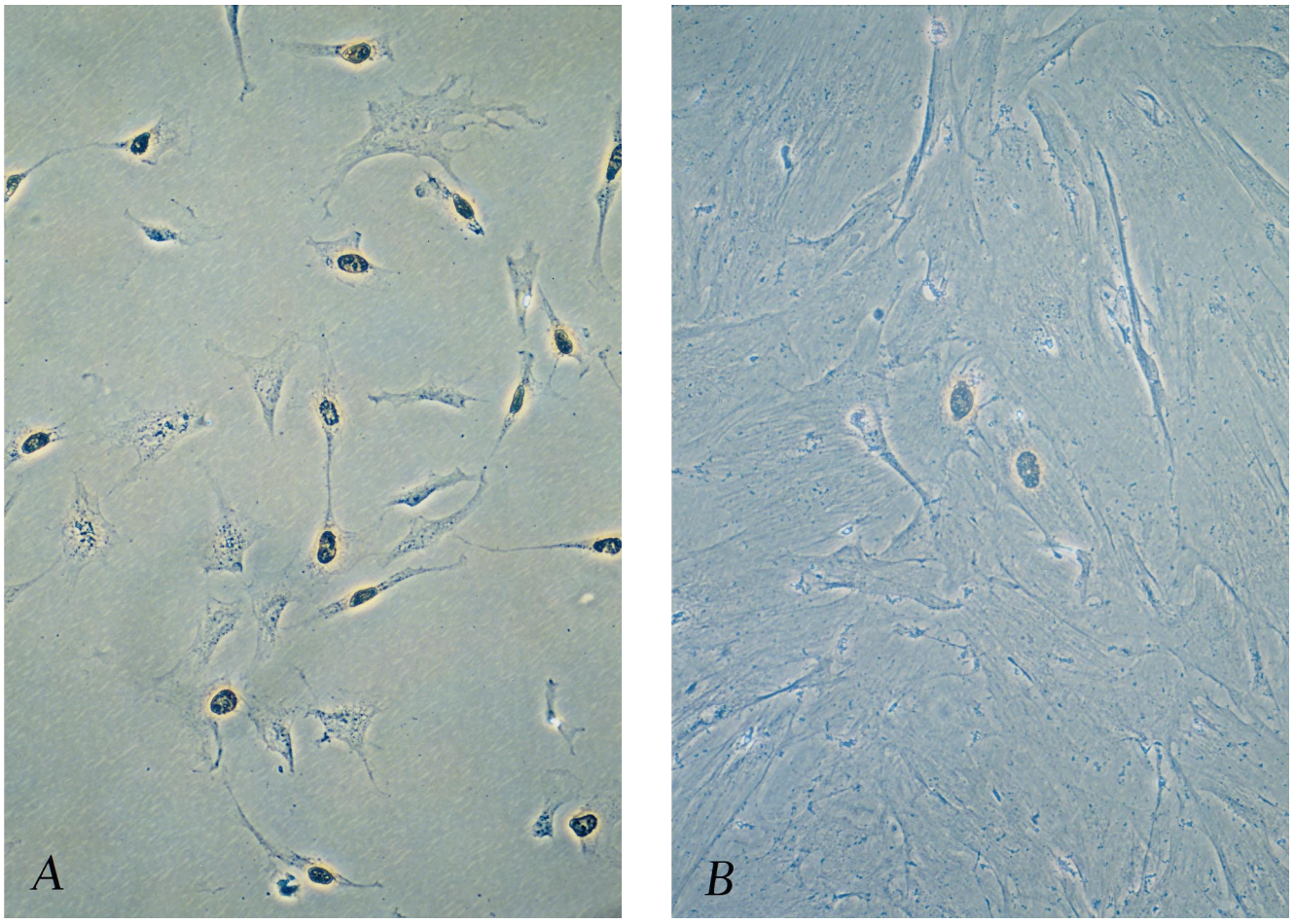

Fig. 2. Indirect immunostaining of BrdU. The incorporation of BrdU shown as brown color was recognized in the nucleus. (A) 5th day of culture. (B) 14th day of culture.

at 72°C for 1 min. In parallel, PCR reaction was performed with primers coding for the house keeping gene, β-actin as internal standard. An aliquot of each PCR reaction was analyzed by an electrophoresis in a 2.0% agarose gel containing ethidium bromide.

3. Results

3.1. Ca^{2+} current in stellate cells

The cells were allowed to stand for several minutes to equilibrate the cell interior with the pipette solution. The holding potential was usually set at −70 mV. Representative currents responded to depolarizing step pulses are shown in Fig. 1. Inward current induced with a depolarizing pulse of 0 mV increased rapidly and decayed slowly during 400 ms (Fig. 1A, open circle). The inward current following depolarization was diminished with the addition of 0.5 mM Cd^{2+}, a dihydropyridine-type Ca^{2+} channel blocker, to the superfusate (Fig. 1A, closed circle) and was also decreased in the absence of extracellular Ca^{2+} to nominally zero (data not shown) as expected for Ca^{2+} current. Representative inward Ca^{2+} currents elicited by 400 ms step depolarization in 10-mV increments from a holding potential of −70 mV to +20 mV, Cd^{2+}-sensitive currents calculated from the difference in currents before and after the application of Cd^{2+}, are superimposed in Fig. 1B, and a typical relationship between the maximal current and applied voltage is shown in Fig. 1C. Inward Ca^{2+} current appeared with depolarizing pulses over −40 mV and reached a maximum at −10 mV. The maximal current density varied from cell to cell (range between 0.39 and 7.26 pA/pF) with a mean

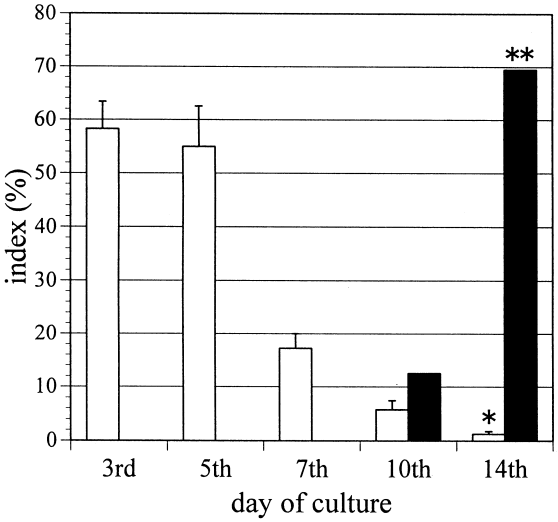

Fig. 3. BrdU labeling index and Ca^{2+} current index during culture. The BrdU index was studied in 100 cells in each experiment (□). Ca^{2+} current index (■) was the ratio of current-positive cells per total studied cells (0/14 at the 7th, 2/16 at the 10th and 16/23 at the 14th day of culture, respectively). Amplitude of less than 10 pA was eliminated as negative current. Vertical axis is ratio of BrdU positive cells and incidence of Ca^{2+} current (%). Horizontal axis is the day of culture. $*P<0.01$ (one-way ANOVA), $**P<0.01$ vs. 10th day (2×2 Chi-square).

value of 1.9 ± 0.5 pA/pF at the 14th day of culture (mean ± S.E.M., $n=16$). The membrane capacity in the cells associated with inward Ca^{2+} current ranged from 31 to 155 pF (mean was 80 pF). Inward Ca^{2+} currents (voltage-activated Ca^{2+} currents) at the 14th day of culture were shown in this study, because Ca^{2+} currents were observed frequently in the cells cultured for more than 10 days.

3.2. BrdU staining

The staining of BrdU in stellate cells were shown in Fig. 2. At the 5th day of culture, many cells showed the incorporation of BrdU (Fig. 2A). On the other hand, cells proliferated and spread on glass showed few BrdU staining at the 14th day of culture (Fig. 2B).

3.3. BrdU labeling index and Ca^{2+} current index

After inoculation onto glass, stellate cells showed an active proliferation. The BrdU labeling index obtained from four rats (mean ± S.D., %) was 58 ± 5 and 55 ± 8 of cells at the 3rd and 5th day of culture, respectively, then the index decreased significantly to 17 ± 3, 6 ± 2 and 1.2 ± 0.5 of cells at the 7th, 10th and 14th day of culture, respectively (Fig. 3). On the other hand, no Ca^{2+} current was observed in cells cultured for less than 9 days; however, it then was detected increasingly in a time-dependent manner. The Ca^{2+} current index obtained from four rats increased significantly to 69.5% of studied cells at the 14th day of culture compared to 12.5% of studied cells at the 10th day of culture (Fig. 3). Taken together, Ca^{2+} current was detected in inverse proportion to the cell proliferation in cultured stellate cells.

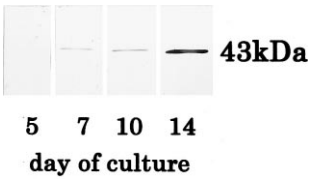

Fig. 4. Western blot analysis for α-smooth muscle actin. The expression of α-smooth muscle actin was seen at 43 kDa from the 7th day of culture. The amount of α-smooth muscle actin increased in a time-dependent manner.

3.4. Detection of α-smooth muscle actin

Western blot analysis was performed to detect α-smooth muscle actin in cultured stellate cells. An apparent band was not detected in cells cultured for less than 5 days, however; a single band at 43 kDa was detected from the 7th day of culture

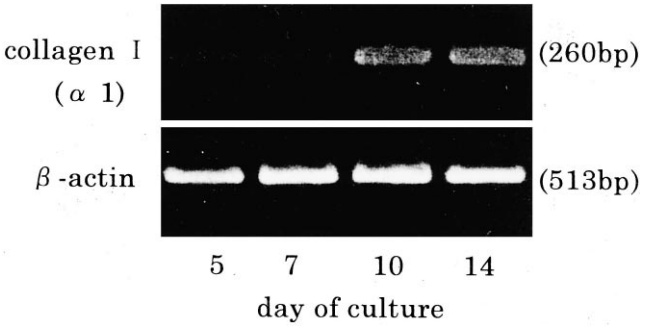

Fig. 5. RT-PCR analysis of collagen type I (α1) mRNA in cultured stellate cells using the specific primer pairs predicted to amplify fragment size on the right. Upper: collagen type I mRNA; lower: β-actin as internal control. The PCR products were detected by electrophoresis on a 2% agarose gel as describes in experimental procedures.

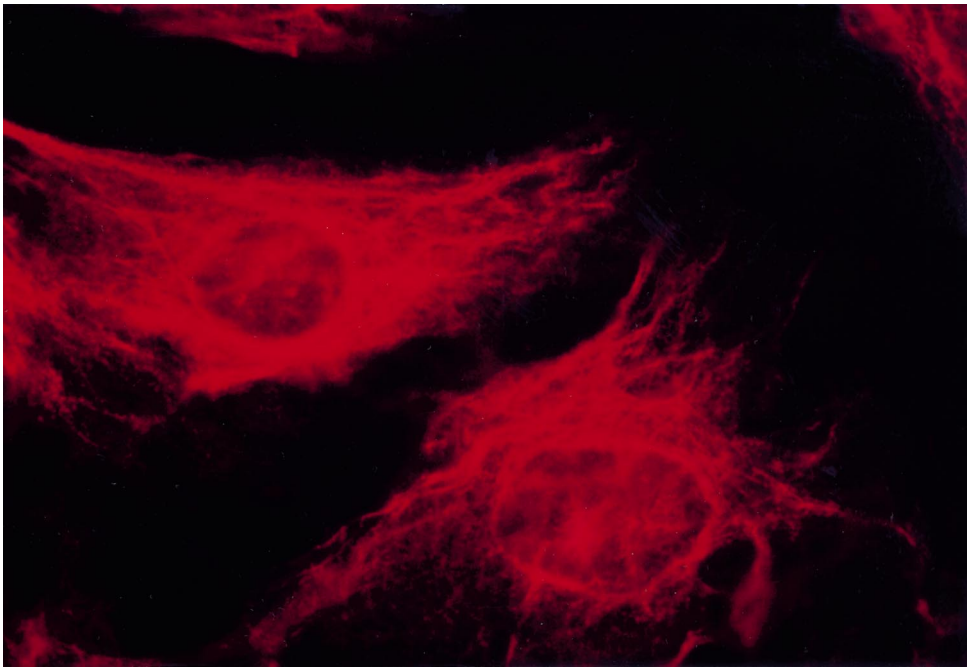

Fig. 6. Immunocytochemistry for desmin in cultured stellate cells. Desmin was still recognized in the cytoplasm at the 14th day of culture (magnification ×50).

and the quantity of α-smooth muscle actin increased in a time-dependent manner (Fig. 4).

3.5. Detection of collagen type I mRNA

The results from RT-PCR of collagen type I mRNA in cultured stellate cells are shown in Fig. 5. The expression of collagen type I mRNA was recognized in cells cultured for more than 10 days. β-Actin mRNA, a control to demonstrate the equivalent amounts of cell RNA, was used for cDNA synthesis and was detected in all samples.

3.6. Immunocytochemistry

Desmin, a marker for identification of stellate cells, was still positive at the 14th day of culture (Fig. 6).

These results indicated that voltage-activated Ca^{2+} current appeared in activated stellate (myofibroblast-like) cells following an active cell proliferation.

4. Discussion

We already reported the existence of voltage-activated Ca^{2+} channels which are activated by transforming growth factor-β1, a factor for hepatic fibrosis, in cultured stellate cells using pharmacological technique [13]. The suppression by Ca^{2+} channel antagonists of the sustained phase of angiotensin II- and thrombin-induced increase in intracellular calcium concentration is also evidence of the contribution of Ca^{2+} channels on the agonist-induced contraction in activated stellate cells [18]. Furthermore, we had reported that voltage-activated Ca^{2+} channel in stellate cell was L-type using the patch clamp technique [19]. This technique is very useful and a direct strategy to evaluate a proper character of not only Ca^{2+} channels but also other ion channels such as K^+ channels.

Interestingly, Ca^{2+} current was detected in activated stellate cells following cell proliferation in this study. After inoculation of cells onto glass coverslips, stellate cells showed an active proliferation. During this phase, no cells showed Ca^{2+} current, and neither α-smooth muscle actin nor collagen type I mRNA were recognized. After the 7th day of culture, cell proliferation was down-regulated significantly, then Ca^{2+} current became apparently accompanied with the production of α-smooth muscle actin and collagen type I mRNA. α-Smooth muscle

actin in stellate cells was reported to be detected strongly 7 days after isolation [20]. These activated stellate cells still showed desmin as a character for myogenic cells. Hence, it is obvious that activated stellate (myofibroblast-like) cells possess L-type voltage-activated Ca^{2+} current. Stellate cells are known to proliferate around damaged sites and to undergo an activated change from the quiescent fat-storing phenotype to the myofibroblast-like phenotype in response to alcohol [21] and extrinsic toxic substances which induce chronic liver damage [2]. Stellate cells observed in this study showed a spontaneous conversion to a myofibroblast-like phenotype during culture as reported previously [22] following an active proliferation.

Furthermore, activated stellate cells were reported to contract with endothelins [14]. A conversion to myofibroblast-like cells is a very important factor in various type of liver damage. Taken together, L-type voltage-activated Ca^{2+} channel of activated stellate cells could be involved in the regulation of hemodynamics and pathophysiological remodeling (fibrosis) in liver.

The role of the Ca^{2+} channel on the production of extracellular matrix in activated stellate cells is unknown. Ca^{2+} channel blockers were reported to abolish collagen deposition in human vascular smooth muscle cells and fibroblasts in terms of atherosclerosis [23], and mammalian fibroblasts were known to retard extracellular matrix production with Ca^{2+} antagonists in connective tissue [24]. Glomerulosclerosis induced by renal mesangial cells has been demonstrated to be inhibited by calcium channel antagonists [25]. Taken together, it is suggested that Ca^{2+} channels contribute to the production of extracellular matrix. Hence, an evaluation of the effect of Ca^{2+} channel antagonists on liver fibrosis might be helpful to consider the contribution of Ca^{2+} channels in stellate cells.

Acknowledgements

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