Biochemical and Biophysical Research Communications 430 (2013) 1240–1245

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

p38MAPK and Rho-dependent kinase are involved in anoikis induced by anicequol or 25-hydroxycholesterol in DLD-1 colon cancer cells

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ARTICLE INFO

Article history:
Received 14 December 2012
Available online 22 December 2012

Keywords:
Anoikis
Anicequol
25-Hydroxycholesterol
p38MAPK
Rho-associated
Coiled-coil containing protein kinase (ROCK)

ABSTRACT

Anchorage-independent growth is evidence of the malignant transformation of cells. We previously reported the characterization of anicequol, a novel inhibitor of the anchorage-independent growth of tumor cells, and here we show that the effects of 25-hydroxycholesterol (25-HC) on colon cancer cells were very similar to those of anicequol. By analyzing the effects of inhibitors and performing RNA interference experiments, we found that p38 mitogen-activated protein kinase (p38MAPK) was involved in anicequol- and 25-HC-induced anoikis in DLD-1 cells. In addition, Rho-associated, coiled-coil containing protein kinase (ROCK) was also associated with anoikis induced by anicequol or 25-HC. Taken together, our findings suggest that activation of the p38MAPK and ROCK pathways might provide a new therapeutic strategy against cancer, and raise the possibility that tumor metastasis is influenced by 25-HC under physiological conditions.

1. Introduction

Anoikis refers to apoptosis that is induced by the loss of cell adhesion to the extracellular matrix (ECM) [1–3]. It is associated with tissue homeostasis, development, and disease. Cancer cells are particularly resistant to anoikis [4]. Gain of resistance to anoikis is accomplished by different strategies that converge on survival signals and/or apoptotic signals [1]. Anoikis functions as a physiological barrier to metastasis, whereas resistance to anoikis allows cancer cells to survive in the circulation and gain access to other organs [5,6]. Thus, elucidation of the mechanisms underlying anoikis resistance should be beneficial in terms of drug design and the development of novel cancer therapies.

Cells can avoid anoikis by gaining constitutive activity with respect to certain survival pathways; these pathways induce autocrine growth factor loops or stimulate neighboring cells in a paracrine manner [7,8]. Changing the pattern of integrin expression is also known to be a strategy to avoid anoikis [1–3]. In addition, reactive oxygen species (ROS) are key players in anoikis resistance because they transduce prosurvival signals [9,10]. Hypoxia-mediated increases in ROS production might also enable cells to overcome anoikis by means of the downregulation of proapoptotic factors [9]. Epithelial–mesenchymal transformation (EMT) is another strategy to escape anoikis. The upregulation of several transcription factors is critical for the success of EMT and for overcoming the apoptotic pathways [11–14].

We previously isolated anicequol, which is a novel inhibitor of the anchorage-independent growth of tumor cells [15]. Anicequol was identified from *Penicillium aurantiogriseum Dierckx* TP-F0213. Its structure was determined to be (3β, 5α, 7β, 11β, 16β, 24S)-16-acetoxy-3,7,11-trihydroxy-ergost-22-en-6-one, and it is a sterol (oxysterol) (Fig. 1A). Thus, anicequol is predicted to function by mimicking endogenous oxysterols. Oxysterols induce many different biological processes, the most important being apoptosis [16]; this therefore, raises the possibility that some oxysterols act to induce anoikis.

Here, we demonstrate that p38 mitogen-activated protein kinase (p38MAPK) and Rho-associated protein kinase (ROCK) are involved in anoikis which is induced by anicequol or 25-hydroxycholesterol (25-HC) in DLD-1 cells. p38MAPK and ROCK inhibitors reduced the degree of anoikis induced by anicequol and 25-HC. Silencing of the p38MAPKalpha gene also reduced anicequol- and 25-HC-induced anoikis; in addition, p38MAPK and ROCK shared the same anoikis induction pathway. These results indicate that anicequol- and 25-HC-induced anoikis are regulated by a novel pathway, and suggest that 25-HC has an inhibitory role with respect to the invasiveness of colon cancer cells under physiological conditions.

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http://dx.doi.org/10.1016/j.bbrc.2012.12.067

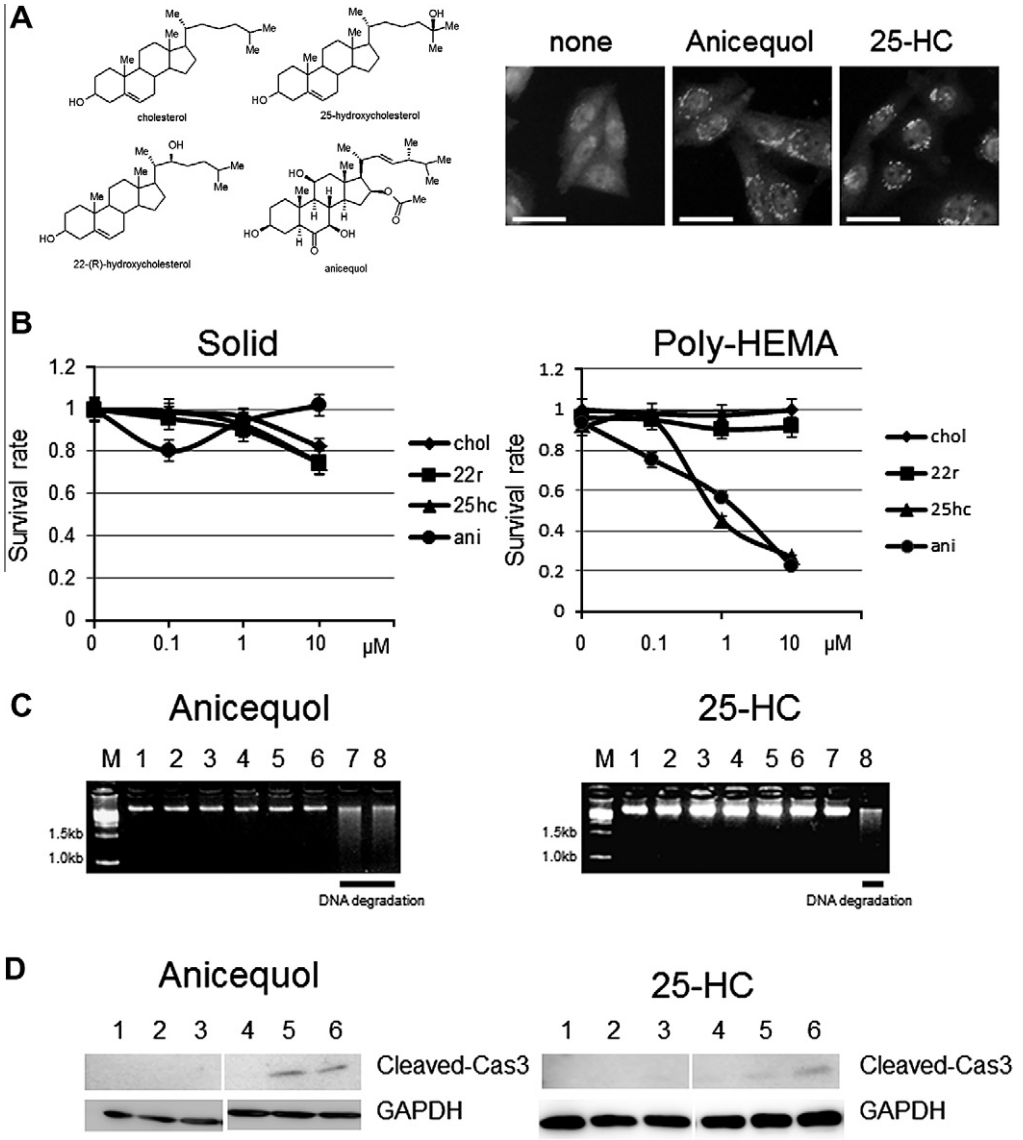

Fig. 1. Anoikis induction by anicequol and 25-hydroxycholesterol (25-HC) in DLD-1 cells. (A) Structure of oxysterols (left) and effect of anicequol on OSBP localization (right panels). CHO cells were treated with each compound (10 μM) for 8 h and processed for indirect immunofluorescence using anti-OSBP polyclonal antibody. Bar = 25 μm. (B) The effects of oxysterols on anchored or nonanchored DLD-1 cells. The survival rate of cells treated with DMSO only (control cells) was designated 1.00. Each plot represents the means of three independent experiments (triplicate); bars indicate SE. (C) Anicequol (left) and 25-HC (right) induced nucleosomal DNA fragmentation in nonanchored DLD-1 cells in a concentration-dependent manner (M: marker; lane 1: 0 μM, anchored; lane 2: 0.1 μM, anchored; lane 3: 1 μM, anchored; lane 4: 5 μM, anchored; lane 5: 0 μM, nonanchored; lane 6: 0.1 μM, nonanchored; lane 7: 1 μM nonanchored; lane 8: 5 μM, nonanchored). Cells were treated with anicequol or 25-HC for 48 h. Cytosolic DNA was analyzed by agarose gel electrophoresis as described in Section 2. (D) Anicequol (left) and 25-HC (right) induced caspase-3 cleavage in nonanchored DLD-1 cells in a concentration-dependent manner (lane 1: 0 μM, anchored; lane 2: 0.1 μM, anchored; lane 3: 1 μM, anchored; lane 4: 0 μM, nonanchored; lane 5: 0.1 μM, nonanchored; lane 6: 1 μM nonanchored). Cell lysates were electrophoresed through 12.5% SDS–PAGE, and then subjected to immunoblotting using anti-cleaved caspase-3 and anti-GAPDH antibodies.

2. Materials and methods

2.1. Antibodies and reagents

Anti-GAPDH antibody was purchased from Chemicon, and other antibodies were purchased from Cell Signaling Technologies. Anicequol was isolated as described previously [15]. Y27632 was purchased from Wako Pure Chemical Industries. The other chemical reagents were purchased from Sigma.

2.2. Measurement of anchorage-independent growth (MTT assay)

Human colon cancer cell lines (DLD-1, HT-29 and KM-12) were grown in RPMI1640 medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator. Exponentially growing cells were trypsinized and resuspended in fresh medium and then plated onto poly-(2-hydroxyethyl methacrylate) (poly-HEMA)-coated (suspension culture) or uncoated (attached culture) plates. Poly-HEMA-coated plates were prepared as described previously. Cell growth was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) as described previously [17].

2.3. DNA fragmentation assay

Cells were seeded in poly-HEMA-coated or uncoated 35-mm dishes and incubated for 24 h. They were treated with the stated reagents for 48 h, washed with PBS, and lysed with 10 mM Tris–HCl (pH 7.4) 10 mM EDTA, and 0.5% Triton X-100. RNA was digested with RNase (0.1 mg/ml at 37 °C for 1 h) followed by proteinase K treatment at 50 °C for 2 h. DNA was extracted with a mixture

of phenol, chloroform, and isoamyl alcohol (25:24:1). The extracted DNA was then precipitated with 2-propanol, run on a 2% agarose gel, and visualized by staining with ethidium bromide.

2.4. Immunoblot analysis

Cells were seeded as above, treated with the stated reagents for 48 h, and washed with cold Tris-buffered saline (TBS). The cells were then lysed on ice in lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 50 mM NaF and protease inhibitors). Total protein content was measured using a BCA Protein Assay Kit (Pierce). Equal amounts of protein were electrophoresed through SDS–PAGE, immunoblotted, and detected by enhanced chemiluminescence (ECL plus, GE Healthcare).

2.5. RNA interference assay

DLD-1 cells were transfected with siRNAs (50 nM) using Lipofectamine 2000 (Invitrogen). Silencer Negative Control #1, human p38MAPKalpha-specific siRNA (Applied Biosystems) were used. After incubation for 24 h, cells were trypsinized and resuspended in fresh medium and then plated onto poly-HEMA-coated plates. After incubation for 48 h, cell growth was determined using the MTT assay as above.

2.6. Immunofluorescence analysis

CHO cells were treated with compounds at indicated concentrations, for 8 h, and then subjected to microscopic analysis. Indirect immunofluorescent experiment for OSBP was performed essentially as same as described [18]. Cells were then visualized using a conventional fluorescence microscope (OLYMPUS IX70, Japan) equipped with fluorescence digital CCD camera (KEYENCE, VB-6010, Japan).

3. Results

3.1. Anoikis of DLD-1 cells was induced by anicequol and by 25-hydroxycholesterol

We earlier reported that anicequol inhibits the anchorage-independent growth of DLD-1 cells [15]. As shown in Fig. 1A, anicequol has quite similar structure to those of oxysterols, and here we demonstrated that anicequol treatment induced translocation of oxysterol binding protein (OSBP) we well as 25-HC in CHO cells. OSBP translocation by anicequol was also observed in DLD-1 cells (data not shown), and these results suggest that pharmacological effects of anicequol will be similar to 25-HC. Furthermore, we found that the growth of DLD-1 cells in poly-HEMA-coated dishes was also inhibited by 25-HC in a concentration-dependent manner (Fig. 1B, right). In contrast, cholesterol or 22-(R)-hydroxycholesterol did not have this effect. In addition, anicequol and 25-HC induced the fragmentation of genomic DNA (Fig. 1C) and the cleavage of caspase 3 (Fig. 1D), which indicated that both anicequol and 25-HC induced the anoikis of DLD-1 cells. We further determined the profile of anoikis induction by these compounds in 35 different cancer cell lines. We found that anoikis was only induced by anicequol or 25-HC in another colon cancer cell line, namely HT-29 (Fig. 2D). These results suggest that the ability of anicequol and 25-HC to induce anoikis is specific to colon cancer cells. p38MAPK was associated with anoikis induced by anicequol or 25-hydroxycholesterol in DLD-1 cells

To determine the pathways that were associated with the induction of anoikis by anicequol or 25-HC in DLD-1 cells, we checked the phosphorylation status of representative kinases. We found that p38MAPK was phosphorylated in DLD-1 cells cultured in poly-HEMA-coated dishes in the presence of anicequol or 25-HC (Fig. 2A). In addition, HSP27 and ATF2, which are downstream targets of p38MAPK [19,20], were also phosphorylated. Furthermore, this phosphorylation of p38MAPK in response to anicequol or 25-HC was not observed in nonanchored KM-12 cells (anicequol insensitive, Fig. 2B,right) but was observed in nonanchored HT-29 cells (anicequol sensitive) (Fig. 2C). These results suggest that the death pathway of p38MAPK is involved in anoikis of anicequol and 25-HC sensitive colon cancer cell lines [21–23]. To address this hypothesis, we attempted to use SB203580, a popular p38MAPK inhibitor, to elucidate the involvement of p38MAPK in anoikis. However, the treatment of DLD-1 cells in poly-HEMA coated dishes with SB203580 plus sterols resulted in high levels of toxicity. To elucidate this, we knocked down p38MAPKalpha gene expression by using a specific siRNA. Although only the p38MAPKalpha gene was knocked down, the level of total p38MAPK protein was decreased dramatically (Fig. 3A), which indicated that p38MAPKalpha, rather than p38MAPKbeta, gamma or delta, was the dominant p38MAPK in DLD-1 cells. The survival rate of the p38MAPKalpha knockdown cells increased by 1.3-fold relative to that of control cells in the presence of anicequol and by 1.7-fold in the presence of 25-HC (Fig. 3B). These results suggest that p38MAPK, especially the alpha subtype, is associated with anicequol- and 25-HC-induced anoikis.

3.2. ROCK is involved in anicequol- and 25-hydroxycholesterol-induced anoikis

It has been shown that the ROCK inhibitor Y27632 increases the survival rate of embryonic stem (ES) cells, which normally undergo programmed cell death by anoikis [24]. In the present study, we treated nonanchored DLD-1 cells with Y27632 plus anicequol or 25-HC. As a result, we found that Y27632 also decreased anicequol- and 25-HC-induced anoikis (Fig. 4A). The survival rate of Y27632-treated cells increased by 1.6-fold relative to that of control cells in the presence of anicequol and by 1.7-fold in the presence of 25-HC. These results suggest that the Rho/ROCK pathway is associated with not only the anchorage-independent growth of ES cells but also that of colon cancer cells.

Next, we checked the phosphorylation of myosin light chain 2 (MLC2), which is a downstream target of the Rho/ROCK pathway [25]. However, the degree of phosphorylation of MLC2 did not change dramatically in response to anicequol or 25-HC (data not shown), which suggests that MLC2 is not involved in anicequol or 25-HC-induced anoikis.

To investigate the association between p38MAPK and ROCK, we investigated the phosphorylation status of p38MAPK when DLD-1 cells were treated with both the ROCK inhibitor Y27632 and the sterols. As shown in Fig. 4B, Y27632 reduced the amount of p38MAPK phosphorylation that was induced by anicequol or 25-HC. This result suggests that ROCK is linked with p38MAPK and is upstream of it.

4. Discussion

On the basis of the inhibitor and RNAi analysis reported herein, we conclude that anoikis of DLD-1 cells that is induced by anicequol or 25-HC is associated with the p38MAPK and ROCK pathways. In general, ROCK signaling begins with the activation of Rho, a Ras-related small GTPase, which is involved in the regulation of actin-based cytoskeletal structures. ROCK1 and ROCK2 are Rho-activated serine/threonine kinases that indirectly phosphorylate MLC2 [25,26]. The ROCK inhibitor Y27632 inhibited anicequol- and 25-HC-induced anoikis (Fig. 4A) and reduced the amount of

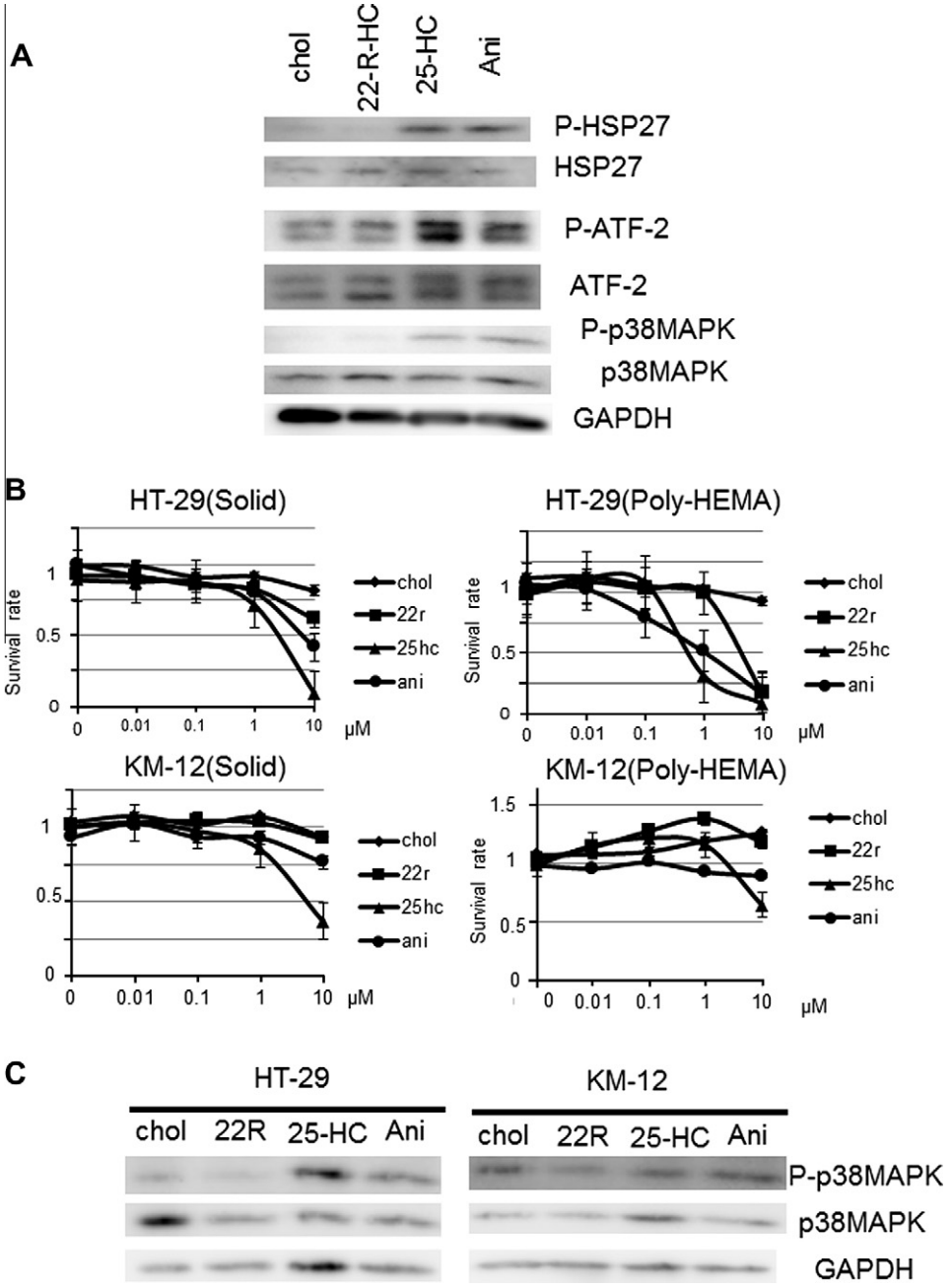

Fig. 2. Activation of the p38MAPK pathway by anicequol or 25-HC in nonanchored DLD-1 cells. (A) Activation of the p38MAPK pathway. Nonanchored DLD-1 cells were treated with cholesterol (1 μM), 22-(R)-hydroxycholesterol (1 μM), 25-HC (1 μM) or anicequol (1 μM) for 24 h. Cell lysates were electrophoresed through 10% SDS–PAGE, and then subjected to immunoblotting using anti-p38MAPK, anti-phospho-p38MAPK, anti-HSP27, anti-phospho-HSP27, anti-ATF2, anti-phospho-ATF2 or anti-GAPDH antibodies. (B) Induction of anoikis by anicequol and 25-HC in colon cancer cell lines (HT-29 and KM-12 cells). The survival rate of cells treated only with DMSO (control cells) was designated 1.00. Each plot represents the means of triplicate wells. Bars indicate SE. (C) The phosphorylation of p38MAPK in nonanchored HT-29 cells, but not KM-12 cells. Nonanchored HT-29 cells or KM-12 cells were treated with cholesterol (1 μM), 22-(R)-hydroxycholesterol (1 μM), 25-HC (1 μM) or anicequol (1 μM) for 24 h. Cell lysates were electrophoresed through 10% SDS–PAGE, and then subjected to immunoblotting using anti-p38MAPK, anti-phospho-p38MAPK, or anti-GAPDH antibodies.

p38MAPK phosphorylation by anicequol and 25-HC. Therefore, ROCK is associated with anoikis and could be a new target of anticancer drugs with respect to metastasis.

In general, oxysterols are considered to be troublesome by products of cholesterol metabolism [27]. They induce many different biological processes but the most important is apoptosis. Oxysterol-induced apoptosis is known to be implicated in the pathogenesis of arteriosclerosis [28], therefore, studies of oxysterols tend to be approached from a negative viewpoint. In this study, 25-HC induced anoikis of DLD-1 and HT-29 cells (Fig. 1). However, 22-(R)-hydroxycholesterol and other oxysterols (7 beta-hydroxycholesterol and 5-cholesten-3beta-ol-7-one) did not have this effect (data not shown), which indicated that 25-HC has a specific effect on DLD-1 and HT-29 cells. Taken together, these results raise the possibility that the 25-HC –induced anoikis is a physiological defense mechanism against the metastasis of colon cancer cells.

In this study, we attempted to identify the direct target of 25-HC and/or anicequol. The functions of 25-HC are known to be: (1) inhibition of HMG CoA reductase mRNA expression [18], (2) activation of the transcription factor LXR (liver X receptor) [29],

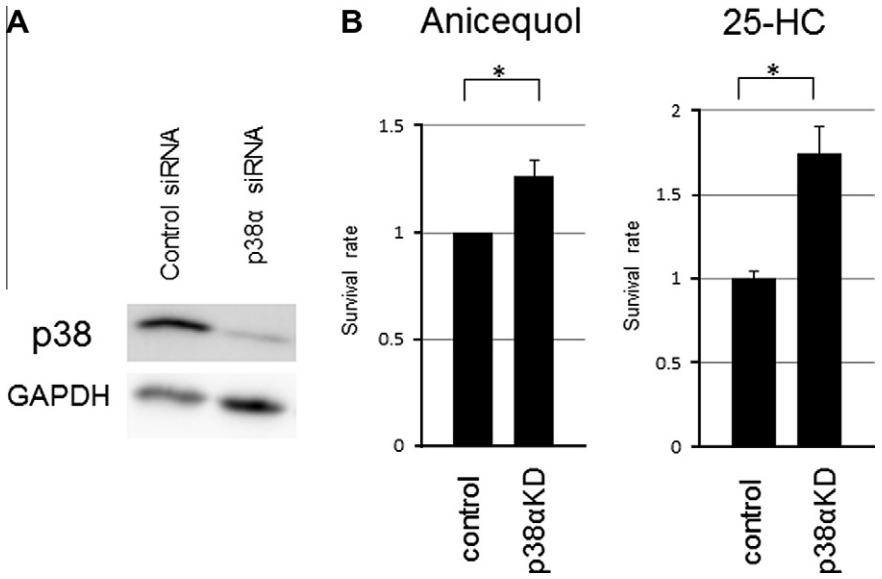

Fig. 3. Downregulation of the p38MAPK pathway inhibits anicequol- and 25-HC-induced anoikis. (A) Knockdown of p38MAPKalpha. The specific siRNA for p38MAPKalpha was transfected into DLD-1 cells for 72 h. Cell lysates were electrophoresed through 10% SDS–PAGE, and then subjected to immunoblotting using anti-p38MAPK or anti-GAPDH antibodies. (B) The effects of p38MAPKalpha knockdown on anicequol (1 μM)-induced anoikis (left) and 25-HC (1 μM)-induced anoikis (right) in nonanchored DLD-1 cells. The survival rate of cells that had been transfected with the negative control siRNA was designated 1.00. Each graph represents the means of three independent experiments (triplicate). Bars indicate SE. $^{*}p < 0.01$ (Student's *t*-test).

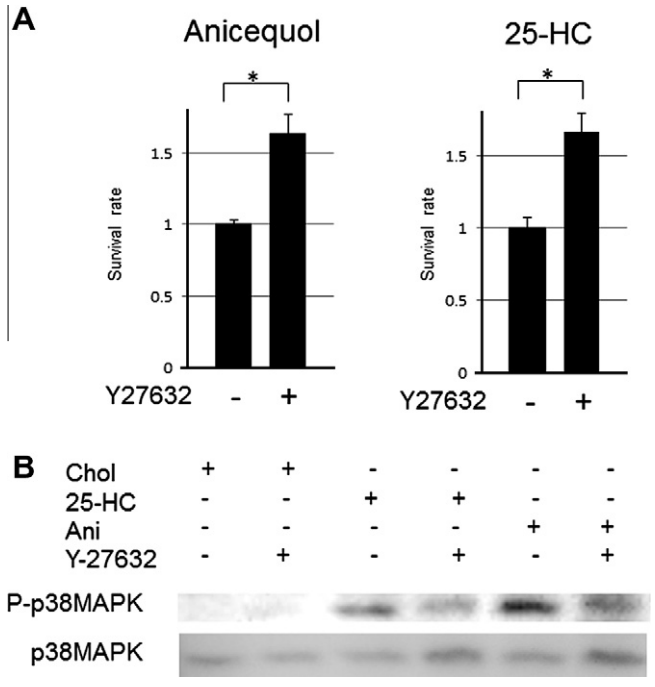

Fig. 4. Downregulation of the ROCK1 pathway inhibits anicequol- and 25-HC-induced anoikis. (A) The effects of Y27632 (10 μg/ml) treatment on anicequol (1 μM)-induced anoikis (left) and 25-HC (1 μM)-induced anoikis (right) in nonanchored DLD-1 cells. The survival rate of cells that had not been treated with Y27632 (control cells) was designated 1.00. Each graph represents the means of three independent experiments (triplicate). Bars indicate SE. $^{*}p < 0.01$ (Student's *t*-test). (B) ROCK and p38MAPK partly shared the anoikis pathway. The effects of Y27632 (10 μg/ml) treatment on anicequol (1 μM)- and 25-HC (1 μM)-induced p38MAPK phosphorylation in nonanchored DLD-1 cells. Nonanchored DLD-1 cells were treated with cholesterol (1 μM), 25-HC (1 μM) or anicequol (1 μM) for 24 h in the presence or absence of Y27632 (10 μg/ml). Cell lysates were electrophoresed through 10% SDS–PAGE, and then subjected to immunoblotting using anti-p38MAPK or anti-phospho-p38MAPK antibodies.

(3) binding to OSBPs (oxysterol binding proteins) [30], and (4) induction of apoptosis by the activation of caspase 3 [31]. It is known that activation of the LXR/RXR (retinoid X receptor) heterodimer by oxysterol effectively blocks cholesterol absorption and induces reverse cholesterol transport in peripheral cells [32]. Therefore, we used the LXR agonist LO-901317 in the anoikis evaluation experiments. However, LO-901317 could not inhibit or enhance anicequol- and 25-HC-induced anoikis (data not shown), which suggested that LXR is not associated with this process. Next, we focused on OSBPs, which control ERK 1/2 activation [33]. Overexpression or knockdown of OSBP1 gave unclear results with respect to anicequol- and 25-HC-induced anoikis (data not shown). Thus, the direct target of 25-HC and/or anicequol remains unknown and should be investigated further in future studies.

We found that the p38MAPK pathway is involved in anicequol- and 25-HC-induced anoikis. The p38MAPK pathway is known as a suppressor of cell survival and tumorigenesis [34]. One p38 MAPK pathway is known to trigger apoptosis by means of the loss of the mitochondrial membrane potential [35]. This known apoptotic pathway might be associated with anicequol- and 25-HC-induced anoikis. In addition, cell detachment is reported to trigger the FasL overexpression associated with p38MAPK in intestinal epithelial and mammary gland cells [23,36]. We examined other kinase pathways (Akt, ERK, SAPK/JNK, FAK), but these kinases were not activated by either anicequol or 25-HC (data not shown). Given that the involvement of p38MAPK is a new finding with respect to anoikis, further investigations into the relationship between anoikis resistance of cancer cells and p38MAPK are needed in the future.

Furthermore, we clarified that ROCK and p38MAPK participate in a shared anoikis pathway which is induced by anicequol and 25-HC in DLD-1 cells. The ROCK inhibitor Y27632 reduced the amount of p38MAPK phosphorylation that occurred in response to anicequol or 25HC, which suggests that ROCK lies upstream of p38MAPK (Fig. 4B). These results suggested the possibility of three anoikis pathways that involved: (1) anicequol or 25-HC > p38MAPK > anoikis, (2) anicequol or 25-HC > ROCK > anoikis, and (3) anicequol or 25-HC > ROCK > p38MAPK > anoikis. The ROCK signal is transduced through p38MAPK or other as-yet-unknown molecules.

Anicequol inhibits the anchorage-independent growth of colon tumor cells. Previously, we determined the properties of anicequol

in DLD-1 cells. The IC_{50} of anicequol against anchorage-independent growth was 1.2 μM whereas the IC_{50} against anchorage-dependent growth was 40 μM. These results led to interesting implications about the potential means of anicequol as an anticancer reagent. Studies on the pharmacological and physiological functions of anicequol and on the target of anicequol will be described in a future paper.

Acknowledgments

We gratefully acknowledge helpful discussions of Drs. Naoyuki Nishiya and Kayoko Tsuda. Financial support for this study was provided by Ministry of Education, Culture, Sports, Science and Technology of Japan.

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