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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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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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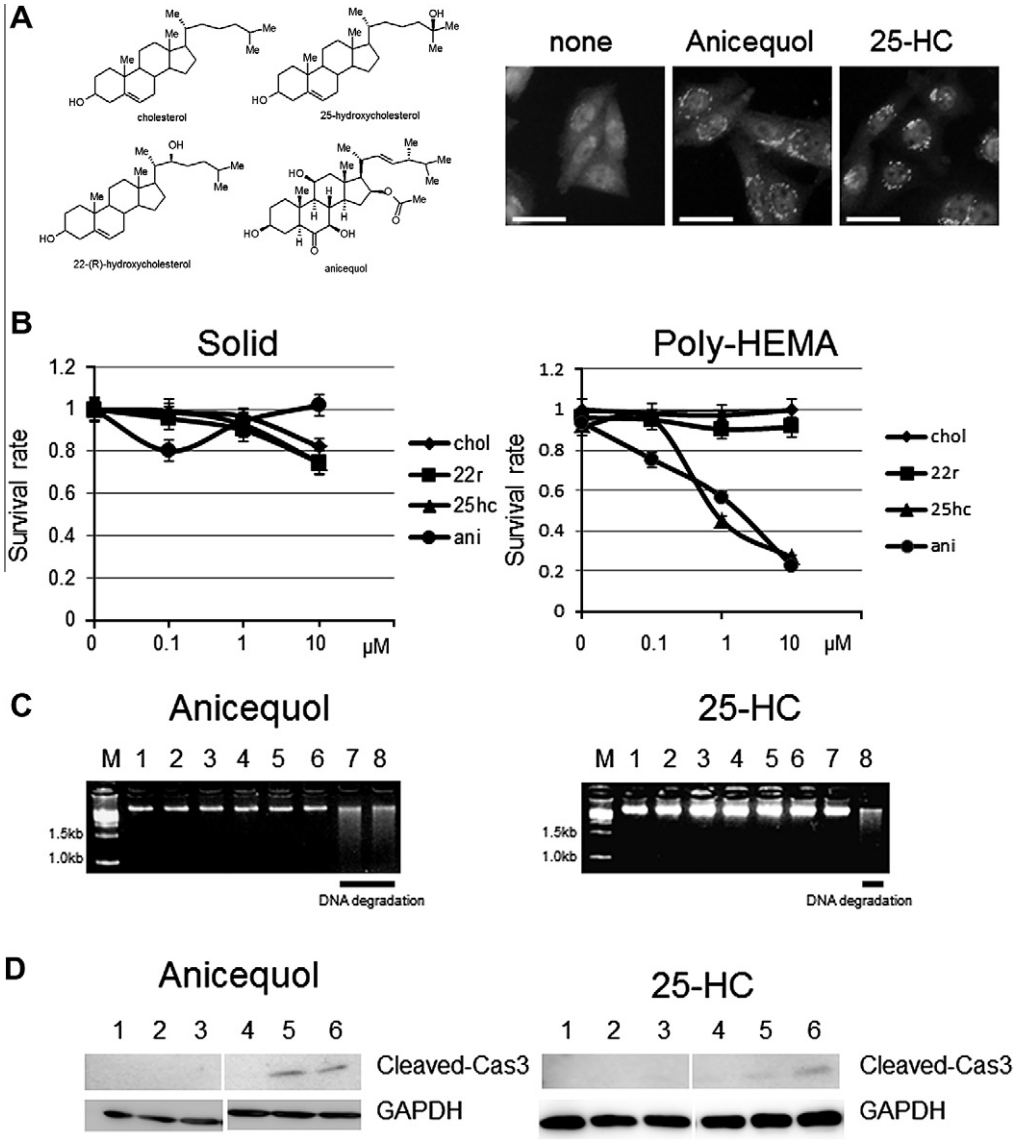

Fig. 1. Anoikis induction by anicequol and 25-hydroxycholesterol (25-HC) in DLD-1 cells. (A) Structure of oxysterols (left) and effect of anicequol on OSBP localization (right panels). CHO cells were treated with each compound (10 μM) for 8 h and processed for indirect immunofluorescence using anti-OSBP 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