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ScienceDirect
Cancer Letters 263 (2008) 53–60
CANCER Letters
www.elsevier.com/locate/canlet

Vitamin K_2 suppresses malignancy of HuH7 hepatoma cells via inhibition of connexin 43

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Received 9 October 2007; received in revised form 6 December 2007; accepted 12 December 2007

Abstract

The anti-cancer potential of vitamin K_2 (VK_2) in hepatoma has gained considerable attention but the underlying mechanisms are unclear. Treatment of HuH7 hepatoma cells with VK_2 produced a normal liver phenotype. Following treatment of cells with VK_2, there was an increase in gap junctional intercellular communication activity, accompanied by up-regulation of connexin 32 (Cx32), dominantly expressed in normal hepatocyte. In contrast, Cx43 expression was inhibited. Moreover, the effect of VK_2 on Cx32 was abolished by over-expression of Cx43. Taken together, we propose that the anti-tumor effect of VK_2 is at least partly due to a decrease in Cx43 promoter activity.

Keywords: Vitamin K_2; Hepatoma; Gap junction; Gap junctional intercellular communication; Connexin 32; Connexin 43

1. Introduction

Vitamin K is a family of structurally similar lipophilic 2-methyl-1,4-naphthoquinones, including phylloquinone (K_1), menaquinones (K_2), and menadione (K_3). VK_2 is well known as a co-factor of γ-glutamylcarboxylase which converts glutamic acid residues into γ-carboxyglutamyl (Gla) residues of blood coagulation factors and bone matrix proteins [1,2]. Meta-analysis of randomized controlled trials showed that supplementation with VK_2 reduces bone loss [3]. More recently, many studies suggest that VK_2 has inhibitory effects against hepatic cancer. In a clinical study, VK_2 prevented development of hepatocellular carcinoma (HCC) cells in female patients with liver cirrhosis [4]. *In vitro* studies indicate that the anti-angiogenic activity and anti-proliferation activity of VK2 might explain the anti-cancer effect of VK_2 [5–7]. However, the precise mechanism by which VK_2 inhibits hepatic cancer is not fully understood.

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doi:10.1016/j.canlet.2007.12.019

Connexin (Cx) is the component protein of the gap junction (GJ) channel, forming structures which mediate intercellular communication. Each GJ is made up of two docked hemichannels (connexons) from neighboring cells, comprising self-assemblies of six Cx proteins [8]. Small molecules (molecular mass of up to 1.5 kDa), ions, second messengers, and metabolites can be directly transferred between neighboring cells through GJs, thereby making possible gap junctional intercellular communication (GJIC) [9,10]. In many tissues, GJIC is responsible for tissue homeostasis such as cardiac conduction and maintenance of CNS function [11,12]. A large number of studies have indicated that Cx genes have a tumor-suppressing effect [13–17]. More than 20 kinds of connexins have been reported and their distributions are tissue specific. Cx32 is dominantly expressed in hepatocytes. Cx32 knockout mice exhibit an increase in the risk of tumorigenesis [18,19]. Cx43 also has tumor-suppressing effects [13,14,16,20,21]; however, it is not expressed in normal hepatocytes and the expression is strongly up-regulated in HCC cells [22,23].

To elucidate the anti-cancer mechanism of VK_2, we focused on the effects of VK_2 on GJIC activity and Cx gene expression in HCC. The treatment of HuH7 cells with VK_2 inhibited proliferation, and induced a normal liver phenotype. We found that GJIC activity in VK_2-treated cell was strongly enhanced. We also showed that VK_2 up-regulated Cx32 at the transcriptional level. On the other hand, Cx43 transcription was inhibited by VK_2-treatment. Finally, we found that Cx43 over-expression abolished the VK_2-induced expression of Cx32 and albumin. These findings suggest that VK_2 indirectly up-regulates Cx32 expression via reduction of Cx43 promoter activity in HuH7 cells, resulting in an activation of GJIC and induction of normal liver phenotype.

2. Materials and methods

2.1. Plasmid construction

The cloning of Cx43 cDNA was described previously (pcDNA3.1-Cx43) [20]. The first PCR product containing the human Cx43 promoter sequence from upstream −1546 to +59 was generated by LA *Taq* polymerase (Takara, Shiga, Japan) using the following primers: 5′-GGAAATAGGATAGCCAACAAGTAAAAGCAA-3′ (forward) and 5′-GGAGGATGAAGTAAAATGAAAAGGCAAG-3′ (reverse) as the first primers. Human genomic DNA was used as the template. Then, the primers, 5′-AAAGCTAGCGCCAACAAGTAAAAGCAAAA-3′ (forward) and 5′-AAAAGATCTTCCTCACGCCTTCCCCCTAA-3′ (reverse) were used as the nested primers to amplify the second PCR product containing the human Cx43 promoter sequence with NheI and BglII restriction enzyme sites. The PCR products were digested with NheI and BglII and ligated into a pGL3-basic promoter (Promega, Madison, WI) using Ligation High (Toyobo, Osaka, Japan). The cloning of the human Cx32 promoter was previously described [24].

2.2. Cell culture and transient transfection

HuH7, human hepatoma cells (RIKEN cell bank, Ibaraki, Japan) were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. For transfection, 2×10^5 cells were plated on 24-well plates. After 48 h, the cells were transfected with 1 μg of pcDNA3.1-Cx43 or the empty vector using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was established by real time-PCR using Ex *Taq* polymerase (Takara) and the following primers: 5′-GGATCGCGTGAAGGGAAGAAGC-3′ (forward) and 5′-GCTGGAAGGTCGTTGGTC-3′ (reverse). These primers can detect transfected rat Cx43, but not endogenous human Cx43 in HuH7 cells.

2.3. Cell growth assay

Ten thousand cells were cultured on 24-well plates with or without VK_2 (Sigma, St. Louis, MO) for 72 h. Trypsinized cells in each sample were counted using a Z1 Coulter Particle Counter (Beckman Coulter, Fullerton, CA) after 24 h, 48 h, and 72 h of cultivation.

2.4. Quantitative RT-PCR

Total RNA was prepared from HuH7 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was subjected to reverse-transcription using ReverTra Ace (Toyobo) and oligo (dT) primer. The cDNA samples were amplified with Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). All the analyses were performed by the comparative Ct Method (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA). Cx43-specific primers were as follows: 5′-ACTCAACAGCCTTATTCAT-3′ (forward) and 5′-CTATGTATCACCTAATGGA-3′ (reverse) for human Cx43 and 5′-GGATCGCGTGAAGGGAAGAAGC-3′ and 5′-GCTGGAAGGTCGTTGGTC-3′ for rat Cx43. The primers for human α-fetoprotein were as follow: 5′-TGCCAACTCAGTGAGGACAA-3′ (forward) and 5′-TCCAACAGGCCTGAGAAATC-3′ (reverse). The other primers for real time PCR were previously described [24].