Kafkas Univ Vet Fak Derg
20 (5): 671-674, 2014
DOI: 10.9775/kvfd.2014.10815

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Osteopontin Expression in Polarized MDCK Cells [1]

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[1] *Preliminary works on this study, presented at 32nd FEBS Congress, 7-12 July, 2007, Vienna - AUSTRIA*

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Makale Kodu (Article Code): KVFD-2014-10815

Summary

The aim of this study is to indicate expression of osteopontin (OPN) in Madin-Darby Canine Kidney (MDCK) cells with different confluences (10, 50, 90, 100%). OPN expression were investigated by western blotting. An increase in OPN expression was observed due to the increased confluency and subsequent initiation of polarization. Expression profiles of flotillin-2 in the same cells were used as a control, since this protein is ubiquitously produced in MDCK cells and its expression rates are independent of confluence and / or polarization. Intracellular distribution of OPN was also monitored by confocal microscopy on preparations immunolabeled with anti-OPN antibodies. Staining patterns have also confirmed increased OPN expression, especially mannose rich isoforms showed an increase dependent on confluency and polarization in MDCK cells. The results were concluded that increased OPN expression during confluency and polarization in MDCK cells. The state of confluence and polarization have significant effects on the mannose rich OPN expression profile.

Keywords: *Osteopontin, Polarization, MDCK cells, Western Blotting*

Polarize MDCK Hücrelerinde Osteopontin Ekspresyonu

Özet

Çalışmada, farklı hücre yoğunluklarındaki (%10, 50, 90 ve 100) Madin-Darby Canine Kidney (MDCK) hücrelerinde, osteopontin (OPN) ekspresyonunun araştırılması amaçlandı. OPN ekspresyonu, western blotting ile araştırıldı. Polarizasyonun başlamasını takiben ve hücre yoğunluktaki artışa bağlı OPN ekspresyonunda artış gözlendi. Hücrelerdeki flotillin-2'nin ekspresyon profili kontrol olarak kullanıldı. Flotillin-2 proteininin ekspresyon hızı, hücre yoğunluğu ve/veya polarizasyona bağımlı olmadan MDCK hücrelerince üretilmektedir. OPN'nin hücre içi dağılımı anti-OPN antikor ile immun işaretleme yapılarak konfokal mikroskopta görüntülendi. MDCK hücrelerindeki hücre yoğunluğuna bağlı artan OPN ekspresyonu, görüntülenen bantlarla teyit edildi. Özellikle OPN'nin mannozdan zengin izoformlarının ekspresyonunun uyarıldığı tespit edildi. Sonuçlara göre, hücre yoğunluğu ve polarizasyona bağlı olarak, MDCK hücrelerinde OPN ekspresyonunda artış gözlendi. Hücre yoğunluğu ve polarizasyon durumu, mannozdan zengin OPN'nin ekspresyon profili üzerine önemli etkisi bulunmaktadır.

Anahtar sözcükler: *Osteopontin, Polarizasyon, MDCK hücreleri, Western Blotting*

INTRODUCTION

Osteopontin (OPN) is a secreted glycoprotein with a multidomain structure and functions characteristic of a matricellular protein [1]. It is highly phosphorylated sialoprotein. OPN interacts with cell surface receptors via arginine-glycine-aspartate sequence (RGD) and non-RGD containing adhesive domains, in addition to binding to components of the structural extracellular matrix. While normally expressed in bone, teeth, kidney and epithelial lining tissues, OPN levels are elevated under conditions of injury and disease like wound healing and inflammation in most tissues studied to date [1,2].

It is expressed at high levels in bony structures of the body. Thus, OPN closely associates with calcified deposits both in normal bone and also in pathologies of ectopic calcification [2,3]. OPN appears to play roles in both the promotion of calcification and mineralization and in the inhibition of calcification. It is found at high levels in

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calcified vascular tissues but in contrast it also acts as an inhibitor of mineralization of bovine aortic smooth muscle cells *in vitro* [4] and ectopic calcification *in vivo* [5].

OPN, also called, early T cell activation gene 1, is a negatively charged acidic hydrophilic protein that is produced by various cell types and participates in diverse physiological and pathological processes, including bone mineralization, oxidative stress, remyelination, wound-healing, inflamation and immunity [6]. OPN has been associated with the progression of numerous types of cancer and this admitted as a marker for cancer malignancy [7-9].

The aim of this study is to indicate expression of OPN in MDCK cells with different confluences.

MATERIAL and METHODS

Cell Culture

Renal epithelial cells of the Madin-Darby Canine Kidney (MDCK) line, type II were used. Cells were grown in Dulbecco-modified Eagle's medium (DMEM) containing 1 g/L glucose, 10% calf serum (FCS) and 1% penicillin-streptomycine at 37°C in a CO_2 incubator. MDCK cells were grown in different cellular confluences (10, 50, 90, 100%).

Reverse Transcription-PCR Analysis

RNA was prepared from confluent MDCK. RNA was reverse-transcribed, and for PCR amplification, 0,5 µl of cDNA was used in 50 µl reactions. The cycling parameters were 95°C for 4 min, 55°C for 1 min, and extension at 72°C for 2 min for 30 cycles, with a final extension period of 2 min at 72°C. 10-µl aliquot of each reaction was electrophoresed through a 2% agarose gel, and the DNA was visualized by ethidium bromide staining under UV light transillumination. The oligonucleotide primers for dog OPNF: GGCATTGCCTACGCCATTCCGA and OPNR: GAGGTGCCTCTCACTGTCCGGGAA were used (SigmaGenosys 2006-07-14).

Confocal Microscopy

MDCK cells grown on cover slips and stained with immunofluorescence method as described [10] for OPN was examined, photographed and confocal imaging on confocal laser scanning microscope (CLSM; Leica, Hannover, Germany). Localization of OPN in MDCK cells by immuno-fluorescence microscopy demonstrated.

Western Blotting of Cell Lysates

Cell lysates were prepared from cell cultures (grown to 10%, 50%, 90% and 100% confluency on 100 mm dishes) by washing each culture dish twice with cold phosphate buffered saline, followed by the addition of 1.000 µl cold lysis buffer (1% Triton-x 100, 1 mM PMSF, 4 mg/ml leupeptin, 4 mg/ml aprotinin, 1 µg/ml pepstatin, 5 µg/ml antipain). Each cell lysate was scraped from the dish, pipetted up and down to complete lysis, and spun at 16.000 g for 10 min to remove in soluble material. Each supernatant was collected and total protein concentration determined by Bradford Protein Assay [11]. Fourty µg of total protein from each cell lysate was used for SDS-PAGE and immunoblotting. Protein gel electrophoresis was done by standard SDS-PAGE methods [12] and immunoblotting by the enhanced chemiluminescence system (Amersham). Cell lysates or conditioned media were fractionated on a denaturing SDS-PAGE gel, electro-phoretically transferred to nylon membrane using a tank blotting system (Hybond P PVDF Transfer Membrane, Amersham) and detected with polyclonal antibody (Biotrend, 100-401-404, raised against human recombinant OPN). The enhanced chemiluminescence detection system (Amersham Corp.) was used to detect immune-reactive bands. Film exposure time was 5 min.

RESULTS

Expression of OPN and flotillin-2 in MDCK cells in different cellular confluences (10%, 50%, 90% and 100%) were shown in *Fig. 1*. Expression profiles of flotillin-2 in the same cells was used as a control, since this protein is ubiquitously produced in MDCK cells and its expression rates are independent of confluence and/or polarization.

Complex-1 and complex-2 forms were same all the cells in different cellular confluency but mannose rich isoforms showed an increase dependent on confluency and polarization. Endo-F and Endo-H tests for isoform of OPN and localization of in MDCK cells were shown in *Fig. 2* and *Fig. 3*, respectively.

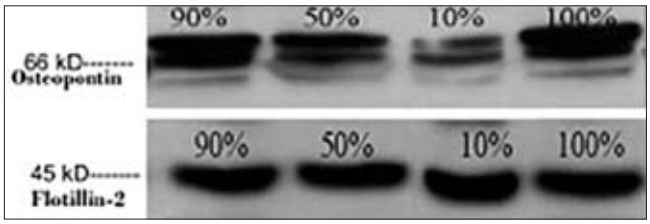

Fig 1. Expression of OPN and flotillin-2 in MDCK cells

Şekil 1. Farklı hücre yoğunluklarındaki MDCK hücrelerinde OPN ve flotillin ekspresyonu

DISSCUSSION

In this study, OPN expression in MDCK cells in different cellular confluences were investigated. Endo-F and Endo-H tests for discovering isoforms of OPN were done *(Fig. 2)*. 2nd band is predominant form (complex-1) and was observed at 66 kDa. 1st band is mannose rich form, 3rd band is complex-2 form. It is thought that complex 1 and 2 isoforms are phosphorylated forms and/or includes O-linked oligosaccharides.

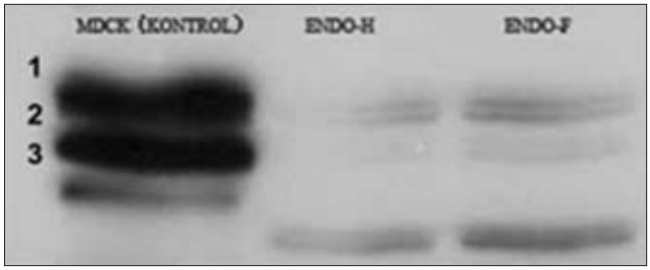

Fig 2. OPN isoforms in different cellular confluences (with Endo-H and Endo-F)

Şekil 2. Farklı hücre yoğunluklarındaki OPN izoformları (Endo-H ve Endo-F ile)

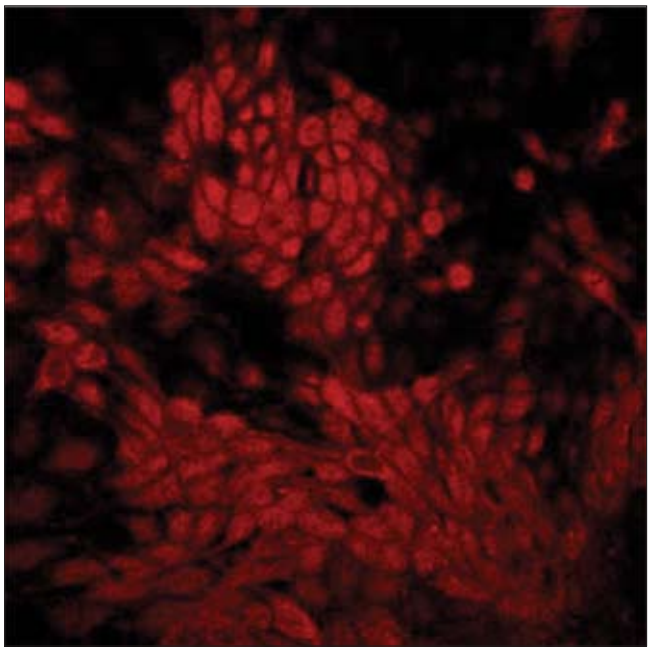

Fig 3. Localization of OPN in MDCK cells

Şekil 3. MDCK hücrelerinde OPN lokalizasyonu

The phosphorylation and dephosphorylation of OPN is an important regulatory mechanism, particularly with regard its role ossification processes. OPN can either faciliate or inhibit ossification depending on phosphorylation state of the protein [13,14].

Malyankar et al.[15] tested the effects of angiotensin 11, basic fibroblast growth factor (bFGF), transforming growth factor/31 (TGF/31), epidermal growth factor (EGF) and insulin like growth factor (IGF), important renal cytokines, on osteopontin regulation in cultured NRK52E cells, a rat renal epithelial cell line. They found that NRK52E cells constitutively express low levels of OPN mRNA and protein. TGF1 and EGF are potent inducers of OPN mRNA and protein in these cells. mRNA stability and nuclear run on assays suggest that induction of OPN expression by TGF1 and EGF is increased via transcription of the OPN gene. The predominant form of osteopontin observed under these conditions had an apparent molecular weight of 66 kDa, consistent with the size previously reported in rat kidney and smooth muscle cells. However, in the TGFf31 treated cells, an additional immunoreactive band was observed. According to Malyankar et al.[15], this band is represent a differentially phosphorylated, glycosylated or spliced isoform of OPN.

Ruutu et al.[16] indicated that numerous genes expression were altered in different confluence states.

At *Fig. 1*, OPN expressionin different cellular confluences were demonstrated. Although, predominant and complex-2 forms are same all the cells in different cellular confluency, mannose rich isoforms showed an increase dependent on confluency and polarization.

In the same cells sometimes OPN can increase some substances but sometimes it can decrease. OPN indicates opposite effects *in vivo* or *in vitro* conditions. This event may depend on transcription of different isoforms of OPN.

The addition of the protein OPN resulted in an increase in the deposition of calcium oxalate [17] or *in vitro* evidence implicates OPN as one of several macromolecular inhibitors of urinary crystallization with potentially important actions at several stages of CaOx crystal formation and retention [18].

OPN originating from different cellular sources may have differential post-translational modifications and/or may be differentially cleaved, suggesting possible differential functions [19]. OPN is normally found in bone, teeth, kidney and epithelial lining tissues. It is expressed at high levels in bony structures of the body, but also in many tissues, the expression of OPN increased under conditions of injury and disease [2,3].

The results were concluded that increased OPN expression during confluency and polarization in MDCK cells. The state of confluence and polarization have significant effects on the mannose rich OPN expression profile.

In order to explain functions of OPN, it is necessary that making more researches about structures and modifications of OPN isoforms. It is necessary to determine the relation between OPN isoforms and diseases.

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