LABORATORY STUDY

Is there any role of epithelial to mesenchymal transition in the pathogenesis of contrast nephropathy?

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ABSTRACT

Aim: Contrast medium-induced nephropathy is one of the major complications of intravenous contrast medium use. But its pathogenesis is unclear. Epithelial mesenchymal transition (EMT) is defined as the transformation of the primer epithelial cells to mesenchymal cells. EMT in tubular cells might cause tubulointerstitial damage. In this study, we investigated whether or not EMT has a role in radiocontrast-induced nephropathy. Radiocontrast medium might be triggering reversible EMT via serum and glucocorticoid-regulated kinase 1 (SGK 1). We investigated the effect of different concentrations of the contrast agent iopromide on human proximal tubule cell (HK-2) culture by measuring the level of SGK1, snail family zinc finger 1 (SNAIL1), connective tissue growth factor (CTGF), and collagen type I alpha 1 (COL1A1).

Methods: We conducted a scratch assay and qPCR. HK-2 cells were cultured in the petri dishes/flasks and starved with serum-free medium. The 40, 20, and 10 mg/mL doses of iopromide were administrated to cells. The scratches were photographed immediately and again at the 20th hour. The levels of gene expression of SGK1, SNAIL1, CTGF, and COL1A1 were measured using the real-time qPCR system at the end of the 24th hour.

Results: Iopromide caused the breaking of intercellular connections, the disappearance of the cobblestone appearance of cells, and the migration of cells at the 20th hour in the scratch assay. It also increased the expression of SGK1, SNAIL1, CTGF, and COL1A1 genes.

Conclusion: Our study concluded that certain important markers of EMT increase in different concentrations of the contrast agent. High osmolality might trigger EMT. The relationship between contrast agent and EMT has not been defined before. Further in vivo and in vitro studies are required.

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Introduction

Radiocontrast agents are commonly used in medical practice. Contrast medium-induced nephropathy (CIN) is one of the major complications of intravenous contrast medium use. CIN is one of the most common causes of hospital-acquired acute renal failure. But its pathogenesis is unclear. Radiocontrast agents affect different areas and different mechanisms in the kidneys. It has direct toxic effect on renal proximal tubulus. It increases blood flow resistance by increasing blood viscosity. The increased viscosity in vessels causes local ischemia and activation of reactive oxygen species, and tubular damage might be observed on cellular level. Besides, it might also cause renal vasoconstriction. This situation increases the renal damage in patients with chronic renal failure.1,2

Generally, reversible acute kidney damage might be observed following contact with the radiocontrast agent. Chronic nephropathy is a process developed with fibrosis. Several pathways are considered to be the cause of this process. Change of tubule-interstitial microenvironment and, especially, epithelial mesenchymal transition (EMT) may trigger fibrosis. A number of mediators released from cells (e.g., growth factors, inflammatory cytokines, chemokines, proteolytic enzymes, complement components, vasoactive amines and peptides, and tissue oxygen tension) might cause this condition. Tubule epithelial cell proliferation and EMT play an important role in the pathophysiology of fibrosis. Although this process is reversible initially, it might turn into chronic fibrosis due to maladaptive healing of repeated damages.3

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TGF-β is generally considered to be the cause of EMT development. TGF-β stimulates connective tissue growth factor (CTGF). CTGF is a matricellular protein. These proteins are released to attack damages and lead to release of other growth factors and proteases. CTGF stimulates extracellular matrix (ECM) production and also triggers biological activity and production of TGF-β. These two growth factors have synergistic effects. Thus, CTGF has an important role in EMT development in renal fibrogenesis.

Snail family zinc finger 1 (SNAIL1) is a transcriptional factor for EMT and fibrosis. TGF-β is major inducer of SNAIL1 in different contexts. SNAIL1 activation in the kidney is sufficient to induce renal fibrosis and renal failure. High levels of SNAIL1 are observed in nephrectomy materials of fibrotic kidneys.

Hyperglycemia, cytoplasmic shrinkage, ischemia, glucocorticoids, and mineralocorticoids stimulate the production of SGK1. SGK1 stimulates renal tubular ion channels and transport. It provides the regulation of renal electrolyte. Increased medullary osmolality in dehydration stimulate SGK1 production and this lead to natriuretic peptide receptors expression and causes natriuresis. SGK1 is important for cell differentiation, proliferation, and life. SGK1 is up-regulated by TGF-β, a key stimulator of fibrosis.

Increased collagen 1 and 3 productions are observed in fibrosis. COL1A1 and COL3A1 gene expressions are increased in epithelial cells following EMT induction in kidneys.

In this study, we investigated whether or not EMT has a role in CIN. Until now, there has been no study of these pathways of EMT in CIN pathogenesis. Hyperosmolarity caused by the radiocontrast medium (CM) might be triggering reversible EMT via SGK 1. We investigated the effect of different concentrations of the contrast agent iopromide on human proximal tubule cell (HK-2) culture by measuring the level of SGK1, SNAIL1, CTGF, and COL1A1.

### Chemicals and cell culture

#### Materials

The radiocontrast medium (RCM) used in our study were iopromide (Ultravist 370, containing 370 mg iodine/mL, Bayer Chemistry, Istanbul, Turkey). TGFβ1 and epidermal growth factor (EGF) (Abcam Cambridge, UK), DMEM/F-12 medium and L-glutamine (Biological Industries, Beit Hemek, Israel), fetal bovine serum and 100U penicillin/0.1 mg streptomycin (Sigma-Aldrich, St. Louis, MO), and immortalized human proximal tubule cell line (HK-2, ATCC CRL-2190TM) (ATCC, LGC Standards GmbH, Wesel, Germany) were provided.

The human proximal renal tubule epithelial cell line (HK-2; American Type Culture Collection (ATCC)) was cultured in DMEM-F12 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% of L-glutamine, and an antibiotic mixture of 100U penicillin/0.1 mg streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

#### Real-time cell analyzer (RTCA) assay

The xCELLigence RTCA (ACEA Biosciences, San Diego, CA) was used to determine the nontoxic contrast medium (CM) doses for HK-2 cell culture. The xCELLigence real-time cell analysis system is used as cytotoxicity assays. It reveals multi-concentration time-dependent cellular response profiles of toxicants on cells. At the beginning of the study E-plates (3 x 10³ cells) were used to prepare the HK-2 cell cultures. The plating process lasted for 24 h. After the plating process, 80, 40, 20, and 10 mg/mL iopromide doses were added onto the cells. Control cells were only subject to the complete medium. The measurements followed 30-min intervals. The cells were monitored for a total of 72 h. Every 24 h, the medium was replaced. The level of iopromide was measured at the end of each 24-h period.

#### Scratch assay

The research question was whether CM caused EMT. For this purpose, a scratch assay method was followed. To culture the HK-2 cells, 35 mm Petri dishes were used. When the cells became 90–100% confluent, the complete medium was removed. The cells were subjected to a 24-h starvation period in serum-free DMEM/F12 medium. Afterwards, the cell monolayer was scraped in a straight line with a 200 µL pipet tip to create a scratch. Then, four Petri dishes were prepared by adding various levels of iopromide as well as transforming growth factor (TGF) and EGF as follows:

- 10 ng/mL of TGFβ1 + 10 ng/mL of EGF
- 40 mg/mL of iopromide
- 20 mg/mL of iopromide
- 10 mg/mL of iopromide

The control Petri dish had only the serum-free medium. Markings were created near the respective scratch to be used as reference points in the imaging process. After treating, the Petri dishes with the substances given above in (a) to (d), the dishes were placed under a phase-contrast microscope (Nikon Eclipse TS100,
Nikon Instruments Europe B.V., Amsterdam, Netherlands) to photograph the images of the scratches at 20× magnification. A 20-h incubation period was administered on the cells before photographing them for the second time at the end of the incubation. Finally, the osmolality of 40, 20, and 10 mg/mL of iopromide and serum-free DMEM/F12 medium were measured to determine the effect of iopromide on the osmolality.

**Real-time qPCR**

For the real-time qPCR analysis, the HK-2 cells were cultured in 25 cm² flasks. Similar to the scratch assay, after the cells became confluent by 90–100%, the complete medium was removed and the cells were starved in serum-free DMEM/F12 medium for 24 h. The control and test groups were created in the same way as the scratch assay. The substances listed in (a) to (d) above were added into the flasks and then the materials were incubated for 24 h. The cells were removed at the end of the 24th hour. Afterwards, the RNA was isolated using a High Pure PCR RNA Isolation Kit (Roche Diagnostics GmbH, Germany) and converted into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Germany). The primers and probes were designed in line with the Universal Probe Library (UPL) program (https://Lifescience.roche.com/webapp/wcs/stores/servlet/CategoryDisplay?tab=Assay+Design+Center&identifier=Universal+Probe+Library&langId) (Table 1). Studying all samples in quartets, the gene expression levels were measured using a LightCycler 480 Real-Time PCR system (Roche Diagnostics GmbH, Mannheim, Germany).

**Statistical analysis**

The REST 2009 relative expression software program (Qiagen GmbH, Germany) was used to analyze the relative gene expression, taking the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as the reference gene, and the control group as the calibrator. $p < 0.05$ was considered significant.

**Results**

**The effect of iopromide on the HK-2 cell proliferation**

Iopromide was administered on the HK-2 cells at different volumes. Following the experiments, it was concluded that only 80 mg/mL of iopromide had a cytostatic effect on the HK-2 cells. On the other hand, no statistically significant difference was observed at other doses of iopromide (Figure 1). At the end of the 24th hour, the IC50 value of iopromide was 71.36 mg/mL. That is why, only 40, 20, and 10 mg/mL doses of iopromide were used in the scratch assay and PCR studies.

![Image of a graph showing cell index values over time for different iopromide concentrations](image)

**Table 1. Sequences of primers and probes used in the real-time qPCR.**

| Gene    | Primer right       | Primer left       | UPL Probe |
|---------|--------------------|-------------------|-----------|
| GAPDH   | GCCCAATACGACCAAATCC| AGCCACATGCCTAGACAC| Probe 60  |
| SNAI1   | ATCTCCGGAGGGGATG   | GCTGGAGACTCTAATCCAGA| Probe 11  |
| SGK1    | TTTCAAGTGGGATG     | GAGAGCTGTGAGACTGGTG| Probe 24  |
| CTGF    | TGGAGATTTTGGGATCGG | CTTGAGCTAGAGAACAGA| Probe 85  |
| COL1A1  | GGAACACCTCGCTCTCCA | GGATTCCTCGACCTAAAG| Probe 67  |

**Figure 1.** The proliferation graph of HK-2 cells administered iopromide. The graph shows the proliferation status for 72 h following a 24-h plating process. The cell index value is a dimensionless parameter. It reflects the biological state of monitored cells. The changes in cell number, cell morphology, or cell viability will lead to a change in the cell index proportionally.
**Scratch assay**

The osmolalities of 40, 20, and 10 mg/mL of iopromide and serum-free DMEM/F12 medium were measured to be 442 mOsm/kgH₂O, 371 mOsm/kgH₂O, 354 mOsm/kgH₂O, and 310 mOsm/kgH₂O, respectively.

It was observed that the intercellular connections were broken, the cobblestone appearance of cells disappeared, and the cells migrated to the scratch at the 20th hour in TGFβ1 + EGF- and iopromide-administered groups. These changes were dose-independent in iopromide-administered groups (Figure 2).

**Gene expression levels**

The levels of mRNA were determined at the end of the 24th hour following the administration. TGFβ1-EGF increased the expression of SNAIL1, CTGF, and collagen type I, alpha 1 (COL1A1) genes (Figure 3(B,C,D)). It was...
also observed to reduce the expression of SGK1 (Figure 3(A)); however, this reduction was not statistically significant. On the other hand, all doses of iopromide statistically significantly increased the expression of SGK1, SNAIL1, and CTGF genes (Figure 3(A,B,C)). Moreover, all doses of iopromide also increased the expression of the COL1A1 gene, while only the 10 mg/mL dose was statistically significant (Figure 3(D)).

**Discussion**

Although the incidence of CIN in the general population is between 2% and 5%, it has been reported that the incidence may increase to 25% in patients with risk factors such as renal failure, diabetes, congestive heart failure, advanced age, and nephrotoxic drugs. CIN pathophysiology is unclear. However, renal medullary hypoxia and direct cellular toxicity have been proposed as two important mechanisms in the pathology of CIN. After exposure to CM, a brief vasodilatation is observed, followed by renal vasoconstriction, and renal blood flow is reduced. Furthermore, it has been suggested that CM accumulating in the renal tubules causes cellular damage due to direct toxic effects and this damage is mediated by reactive oxygen species formation. The increased blood viscosity observed as a result of contrast agent intake, increased osmotic load in the distal tubules and impaired tubuloglomerular “feedback” mechanism contribute to the development of hypoxia. Contrast agent also has direct cytotoxic effects on the tubular cells of the kidney. Reactive oxygen species, obstructions formed by the Tamm-Harsfall proteins within the tubules and the protein damage in the tight junctions of the cells have been demonstrated to play a role in the direct cytotoxic effect and lead to apoptosis in the distal segments of the kidney.

EMT regulates the early development in the living. It is necessary for embryogenesis. Reactivation of EMT is observed in tissue healing and inflammation control as a physiological response. It is pathologically activated in fibrosis and cancer. Both developmental and pathological activation of EMT is observed with similar morphological changes, gene expression, and signal pathways.

When the epithelial cells are transformed into mesenchymal cells, it starts to carry the characteristics of mesenchymal cells and produces mesenchymal markers. There are three types of EMT. Type 1 EMT is related to the embryonic development and organogenesis. Type 2 EMT is related to tissue renewal, wound healing, and fibrosis. It is produced in response to inflammation. It stops as the inflammation regresses. Type 3 EMT is observed in cancer cells. These different EMT types are induced and regulated by the common stimulation cascades, signal transduction pathways, transcription factors, and posttranslational regulations. When the epithelial cells are transformed into the mesenchymal cell, its shape and adhesion and migration characteristics are altered as well. Different biomarkers are recommended to define the different EMT types.

In our study, we used TGF-β as positive control and scratch assay in HK-2 cell culture. We showed the EMT by scratch assay. We observed that the combination of TGFβ1-EGF is more successful for EMT induction in HK-2 cells. Therefore, we used this combination in the scratch assay as a positive control. The cobblestone appearance disappeared, the intercellular gap and the cell size decreased.

![Figure 3. Relative expression levels of SGK1 (A), SNAIL1 (B), CTGF (C), and COL1A1 (D) genes. A fold change was obtained by all groups’ proportion to control group.](image-url)
increased, the cells became spindle-shaped, and they migrated to the scratch. We observed these changes in cell morphology and motility with all doses of iopromide (Figure 2). We did not see a significant difference between iopromide doses. We can say that iopromide caused the EMT based on these findings. To our knowledge, no such a study has been performed to search for the pathophysiology of contrast agent. In addition, we measured SGK1, CTGF, SNAIL, and COL1A1 as markers of EMT.

SGK1 may be stimulated by a hyperosmotic environment or cell damage. It is also stimulated in dehydration and when there is a moderate increase in extracellular salt. In our study, although TGFβ1-EGF had no effect, all doses of iopromide significantly increased the expression of SGK1. We believe that this rise of SGK1 leads to the EMT by increasing the effect of TGFβ1 in HK-2 cells. We believe that hypertonicity caused by CM is an important factor in the pathogenesis of CIN. Extracellular hypertonicity causes water to leave the cells and cell shrinkage in mammalian cells. The SGK1 gene is upregulated by cell shrinkage and regulates the cell volume. In studies on diabetes, the hyper-osmolar condition secondary to glucose stimulates the production of SGK1. SGK1 has been responsible for renal fibrosis in diabetic nephropathy. Stimulators of SGK1 expression include hyperglycemia, cell shrinkage, ischemia, glucocorticoids, and mineralocorticoids. Proximal tubular SGK1 expression is low in the normal kidney. Under normal circumstances, SGK1 is probably not included in the proximal tubular transportation. However, in case of hyperglycemia, it begins to be expressed within the proximal tubule, and tubular glucose transport is stimulated. The hyperosmolar effect of glucose contributes to SGK1 expression. 19, 40, 20, and 10 mg/mL of iopromide in serum-free DMEM/F12 were calculated to be 442 mOsm/kgH2O, 371 mOsm/kgH2O, and 354 mOsm/kgH2O, respectively in our study. CM may be leading to the expression of SGK1 via probable hyperosmolar effect and/or cell damage.

Although the EMT is regulated by different cytokines and growth factors, the main factor in maintaining this process is the TGFβ signal pathway. Morphological changes are observed in cells stimulated by TGFβ1. TGFβ stimulates CTGF. There is a synergistic relationship between CTGF and TGF-β. 20 We have also found that the combination of TGFβ1-EGF and all doses of iopromide significantly increased CTGF expression. Several studies support our results. The overexpression of CTGF has been shown in many fibrotic diseases occurring in different tissues. 21 In experimental studies conducted on rodent renal fibrosis model in diabetes, increased levels of CTGF were detected in cardiac fibrosis and kidney biopsy materials in diabetic rodents. 22–24 SGK1 activates the nuclear factor-κB (NFκB), a transcription factor that facilitates inflammation and fibrosis. CTGF is one of the NFκB-sensitive genes. It has been reported that SGK1-dependent CTGF expression contributes to cardiac fibrosis. 25 CM expresses CTGF in HK-2 cells. This may be an indicator of the appearance of EMT.

SNAIL1 is a transcription factor for EMT and fibrosis. SNAIL induces the expression of mesenchymal marker, whereas it reduces the epithelial markers. SNAIL transcription factors regulate cell survival, adhesion, and migration during EMT. 26 Damage-dependent SNAIL re-activation induces partial EMT in epithelial cells. We found that the levels of SNAIL1 expression were significantly high as a result of the combination of TGFβ1-EGF and for all doses of iopromide. The effect of iopromide was not dose-dependent as in the scratch assay. It has recently been reported that the over-expression of SNAIL1 in the nucleus of epithelial cells causes EMT in vivo. It has been demonstrated in the obstructive rat nephropathy model that SNAIL1 mRNA and protein structures have been up-regulated in the rat tubular epithelial cells. 27

In a study performed on the biopsy materials of patients with kidney transplantation, SNAIL1 was found to be closely related to the fibrogenic, EMT-like response of the tubular epithelium in human renal grafts, which was predictive of graft function loss. 28 Increased collagen 1 and 3 production is observed in fibrosis. Increased COL1A1 and COL3A1 gene expressions were shown in the epithelial cells following EMT induction in the kidney. However, this increase is lower than that observed in the myofibroblasts. 29 In our study, we observed increased COL1A1. Although an increase was observed in all doses of iopromide, the most significant increase was observed in the lowest iopromide dose (10 mg/mL). Lower expression of COL1A1 has been demonstrated in epithelial cells than fibroblasts following 7 days of TGFβ1 stimulation. 30 Our study was terminated in 24 h. The COL1A1 level may be observed to be higher if it were continued longer.

As a result, we have shown for the first time that low-osmolar CM iopromide causes the EMT, even with nontoxic doses. We have also shown changes in cell morphology and mobility by scratch assay and changes in the expressions of some genes and transcription factors involved in the EMT and fibrosis by real-time PCR. High osmolality may be stimulating the EMT. However, these results should be supported by in vivo studies.

Disclosure statement
The authors have no conflicts of interest to disclose.

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