EVALUATION OF THE EXPRESSION OF METALLOPROTEINASES 2 AND 9 AND THEIR TISSUE INHIBITORS IN COLON CANCER CELLS TREATED WITH PHYTIC ACID

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Abstract: Matrix metalloproteinases 2 (MMP-2) and 9 (MMP-9) belong to a zinc dependent family of enzymes that degrade components of extracellular matrix. One postulated mechanism by which inositol hexaphosphate (phytic acid, IP6), an ubiquitous plant component, prevents the activation of MMPs may be due to its ability to chelate minerals. The aim of the study was to evaluate the expression profile of MMP-2, MMP-9 and their tissue inhibitors TIMP-1 and TIMP-2 at the mRNA level in human colorectal cancer cell line Caco-2 treated with IP6. A kinetic study of MMP-2, MMP-9 and TIMP-1, TIMP-2 mRNAs was performed after cells treatment with 1; 2.5; 5 mM IP6 for 1, 6, 12 and 24 h. Quantification of genes expression was carried out using real time QRT-PCR technique. The gene encoding MMP-9 was neither constitutively expressed nor induced by IP6 in Caco-2 cells. IP6 at the concentration of 1 mM evoked increase in MMP-2 transcript level, however, its higher doses (2.5; 5 mM) caused a decrease in this gene expression at 1 h incubation. In 24 h lasting culture along with increasing IP6 concentration, the cells expressed lower and lower MMP-2 mRNA level. In response to 1 and 2.5 mM at 6 h, the cells demonstrated an increased transcriptional activity of the TIMP-2 gene which was accompanied by a decrease in TIMP-1 gene transcription. Treatment of cells with 2.5 mM IP6 at 12 h resulted in a strong increase in both TIMP-1 and TIMP-2 expression. The results of this study show that IP6 modulates MMP-2, TIMP-1 and TIMP-2 genes expression in colon cancer cells at the transcriptional level in a way dependent on its concentration and time of interaction.

Keywords: phytic acid, matrix metalloproteinases, colon cancer, real-time QRT-PCR, mRNA quantification

Tumor metastasis is one of the main causes of poor prognosis in patients with many tumors including colon cancer. The breakdown of collagen type IV, a major component of the basement membranes, is an essential step in the invasion of cancer cells into surrounding tissues as well as metastasis to lymph nodes and distant organs (1).

A family of the matrix metalloproteinase genes encoding zinc and calcium dependent enzymes is capable of degrading the main protein components of extracellular matrix (ECM) and basement membranes (2, 3). Among MMPs, gelatinase subgroup including matrix metalloproteinases 2 (MMP-2) and 9 (MMP-9) digest collagen IV and gelatin (4). Moreover, MMPs participate in the regulation of cytokines, growth factors and adhesive molecules activity as well as in angiogenesis and apoptosis (5). The major cellular inhibitors of matrix metalloproteinases are their tissue inhibitors (TIMPs), supplying a closely regulated mechanism for control, activation and function of MMPs. A family of TIMPs consists of at least 4 members (TIMP-1, -2, -3, -4) (6). All TIMPs can inactivate MMP-9 and TIMP-2 seems to have the highest specific activity. TIMP-1 plays a role during the activation of MMP-9. It also has a capability for inactivation of the active forms of both MMP-2 and MMP-9. TIMP-2 plays a dual role in the regulation of MMP-2 activity. It is essential for the activation of the proenzyme, and on the other hand, it has an integral role in the inactivation of the active form of the enzyme (7, 8).

Inositol hexaphosphate (phytic acid, IP6), a phytochemical present in large amounts in legumes, cereals, oilseeds and nuts, has been reported to possess various health benefits (9, 10). It is strongly negatively charged under physiological conditions (11) and shows great potential for complexing positively charged multivalent cations (12). One of characteristics of IP6 is its antioxidant property related to chelation of Fe^{2+} and suppression of

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hydroxyl radical formation (13). Studies show that this compound is a chemopreventive agent against various types of cancer and plays a role in the control of tumor growth, progression, and metastasis (14). The plausible mechanisms for antineoplastic activity of phytic acid include its ability to function as an antioxidant (15) and mineral binding agent (10, 16). Chelating properties of IP6 are likely to reduce the participation of minerals and trace elements as cofactors in enzyme activities (17).

The aim of the present study was to evaluate the expression profile of MMP-2, MMP-9 and their tissue inhibitors TIMP-1 and TIMP-2 at the mRNA level in human colorectal cancer cell line Caco-2 treated with phytic acid.

**EXPERIMENTAL**

**Cell culture**

The human colon adenocarcinoma cell line Caco-2 (DSMZ, Braunschweig, Germany) was routinely cultured in RPMI 1640 medium (Sigma Aldrich), supplemented with 10% fetal bovine serum (GibcoBRL), 100 U/mL penicillin (Sigma Aldrich), 100 µg/mL streptomycin (Sigma Aldrich) and 10 mM HEPES (GibcoBRL). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cells were treated with 1, 2.5 and 5 mM IP6 (pH 7.4) (Sigma Aldrich) for 1, 6, 12 and 24 h. The IP6-unstimulated Caco-2 cells were used as the control.

**RNA extraction**

Total RNA was extracted from control and IP6 stimulated cells by using TRIZOL® reagent (Invitrogen) according to the producer’s protocol. The RNA extracts were qualitatively checked by electrophoresis in 1.0% agarose gel stained with ethidium bromide. RNA concentration was determined spectrophotometrically, on the basis of absorbance values at a wavelength of 260 nm, using a GeneQuant pro (Amersham Biosciences).

**Real-time QRT-PCR assay**

Transcriptional activity of MMP-2, MMP-9, TIMP-1 and TIMP-2 genes was evaluated by the use of real time QRT-PCR technique with a SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit, Qiagen). The analysis was carried out using an Opticon™ DNA Engine Continuous Fluorescence detector (MJ Research, USA). Oligonucleotide primers specific for MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNAs were designed using Primer Express 2.0 software (PE Applied Biosystems, USA) (Tab. 1). QRT-PCR assay was performed in triplicate for each sample. The thermal profile for one-step RT-PCR was as follows: 50°C for 30 min for reverse transcription and 95°C for 15 min followed by 45 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 45 s, for amplification. After completion of the cycle process, the samples were subjected to temperature ramp from 60°C to 95°C at the rate of 0.2°C/s with continuous fluorescence monitoring for melting curve analysis. β-Actin was used as internal control in each single QRT-PCR for all samples. The mRNA copy numbers of examined genes were determined on the basis of the commercially available standard of β-actin (TaqMan DNA Template Reagent Kit, Applied Biosystems). The obtained results of mRNA copy number were recalculated per mg of total RNA. Finally, specificity of RT-PCR reaction was confirmed by determining the characteristic

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**Table 1. Characteristics of primers used for amplification.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of primers</th>
<th>Length of amplicon (bp)</th>
<th>TM (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>Forward: 5’-TCCACTGTGTGTGGGAACTCA-3’</td>
<td>121</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TTGTCTGCACCCACATCTTT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>Forward:5’ TTCTGCCCCAGCGAGAAGA 3’</td>
<td>101</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Reverse:5’ GTGCAGGCGGGAGATGGATGG 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Forward:5’ GCCAGACGAGAGGTCTCTGCGGATACTTCC 3’</td>
<td>118</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ GCCAGAAGACTGAGGATGTGATG 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Forward: 5’ CCCCAAGCAGGAGAGGTTTCTCGACATCG 3’</td>
<td>100</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ TGGACCAGTGCAGAACCCTTGGAGGCT 3’</td>
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bp – base pair; TM – temperature of melting
Evaluation of the expression of metalloproteinases 2 and 9 and their tissue inhibitors TIMP-1, TIMP-2 in colon cancer cells stimulated with IP6 using real-time RT-PCR technique was determined. Specificity of RT-PCR for the target genes was experimentally confirmed by PAA electrophoresis and the amplimer melting temperatures. For each RT-PCR product of MMP-2, TIMP-1 and TIMP-2 a single peak in melting curve analysis was obtained at the expected temperatures, i.e., 82.4°C ± 0.40 for MMP-2; 80.9°C ± 0.28 for TIMP-1; 81.8°C ± 0.32 for TIMP-2. RT-PCR amplimers of MMP-9 showed incorrect dissociation curve and temperature of melting (83.5°C ± 2.02). Gel electrophoresis revealed the presence of single products of the predicted size of MMP-2 (121 bp), TIMP-1 (118 bp), TIMP-2 (100 bp) and lack of MMP-9 amplimer (data not shown).

The Caco-2 cells did not show the expression of MMP-9 gene either under normal conditions or following treatment with IP6 at all tested concentrations for several interaction times.

The transcriptional activity of MMP-2 was detected at 10^2 copies level both in the control and treated with IP6 cells for 1, 6, 12 and 24 h. At 1 h, MMP-2 gene revealed different expression in the control cells and cultures treated with IP6 (p = 0.0002; ANOVA). In response to 1 mM IP6, Caco-2 exhibited significantly higher expression of this gene than unstimulated cells (p = 0.0117; Tukey test), and conversely, cells incubation with 2.5 mM IP6 (p = 0.0548) as well as 5 mM IP6 (p = 0.0239) resulted in its lower expression. Statistical analysis of the experimental data revealed no significant changes in the MMP-2 gene expression in the cells cultured in the presence of IP6 compared to the control for 6 h (p = 0.2285; ANOVA) and 12 h (p = 0.1040). Considering 24 h lasting cell cultures, the MMP-2 mRNA level was markedly different in unstimulated and IP6-stimulated cells (p = 0.0003; ANOVA). Cells treated with 1 mM (p = 0.0052; Tukey test), 2.5 mM (p = 0.0008) and 5 mM IP6 (p = 0.0005) showed a decrease in MMP-2 transcript level in comparison to control cells (Fig. 1A).

Tissue inhibitors of metalloproteinases 1 and 2 were expressed in unstimulated and IP6-stimulated Caco-2 cells at 10^3 and 10^5 mRNA copies per 1 µg RNA, respectively. There were no statistically significant changes in TIMP-1 as well as TIMP-2 genes expression in the controls and the cells treated with increasing concentrations of IP6 for both 1 and 24 h.
(p > 0.05; ANOVA) (Fig. 1B-C). Comparative analysis of TIMPs mRNA copies per 1 mg of total RNA revealed statistically significantly diverse amounts in the control cells and in all cell cultures incubated with IP6 for 6 h (TIMP-1 p = 0.0053, TIMP-2 p = 0.0060; ANOVA). The treatment of cells with 1 mM (p = 0.0058; Tukey test) and 2.5 mM (p = 0.0117) of IP6 resulted in a decrease in the TIMP-1 mRNA levels compared to the control. The expression of TIMP-1 gene in cells exposed to 5 mM was insignificantly lower than in untreated cells. The transcriptional activity of TIMP-2 gene in response to 1 mM (p = 0.0080; Tukey test), to 2.5 mM IP6 (p = 0.0169) was markedly higher than in control cells and cells incubated with 5 mM IP6 exhibited its higher expression, nevertheless with no significant difference. After 12 h incubation of the cells with phytic acid, there were significantly altered expression of both TIMP-1 (p < 0.0001; ANOVA) and TIMP-2 mRNAs (p = 0.0006). The markedly higher level of inhibitors transcript than in control cells were induced only by 2.5 mM IP6 (TIMP-1 p = 0.0002, TIMP-2 p = 0.0233; Tukey test).

**DISCUSSION AND CONCLUSION**

The anticancer activity of IP6 is one of the most important beneficial activities of IP6. It has been demonstrated to have both preventive and therapeutic effects against various cancers. IP6 exerts its effect in cancer cells by modulating their proliferation and apoptosis, adhesion and metastatic activity (14, 18–23). However, the molecular mechanisms of IP6 action are still under investigation.

In the current study we evaluated the influence of IP6 on the expression of genes encoding matrix metalloproteinases 2 and 9 and their tissue inhibitors 1 and 2 in human colon cancer cells. The MMP-2, TIMP-1 and TIMP-2 mRNAs were detected in both IP6-stimulated and unstimulated cells. The gene encoding MMP-9 was not expressed in Caco-2 cells. MMPs are known to exhibit tissue-specific expression. Unlike MMP-2 and TIMPs, which are constitutively produced by a wide variety of cells, MMP-9 expression occurs only in trophoblasts, osteoclasts, macrophages, migrating keratinocytes and leukocytes and their precursors (6, 7). The lack of MMP-9 expression in Caco-2 culture was observed by others researchers. Kermorgant et al. (24) confirmed the appearance of MMP-2, but no MMP-9 mRNA in Caco-2 by RT-PCR technique. Likewise, Li and Shan (25) revealed lack of MMP-9 transcript in the MMP-2, TIMP-1 and TIMP-2 mRNAs presence. They found the lower expression of MMP-2 than TIMPs, similarly to our study.

The transcriptional activity of MMP-2 and -9 genes is induced by a diverse extracellular stimuli, such as cytokines and growth factors (25, 26). In this study the Caco-2 cells were exposed to phytic acid. IP6 did not activate the transcription of MMP-9, but MMP-2 and TIMPs genes showed time- and dose-dependent expression. The modulation of MMP-2 expression was detected after 1 h and 24 h incubation of the cells with IP6, whereas the significant changes in the transcriptional activity of TIMP-1 and TIMP-2 were observed in 6 h and 12 h culture. IP6 at 1 mM evoked an increase in MMP-2 transcript level, however, its higher doses (2.5; 5 mM) caused a decrease in this gene expression at 1 h incubation. In 24 h lasting culture, along with increasing IP6 concentrations the cells expressed lower and lower MMP-2 mRNA level. In response to 1 and 2.5 mM IP6 at 6 h, an increased transcriptional activity of the TIMP-2 gene was accompanied by a decrease in TIMP-1 gene transcription. Treatment of cells with 2.5 mM IP6 at 12 h resulted in a strong increase in both TIMP-1 and TIMP-2 expression.

Studies on the influence of IP6 on expression of MMP-2 and -9 and TIMPs in human colon cancer cells have not been demonstrated, as yet. Tantivejkul et al. (27) reported that IP6 at 2 mM significantly inhibited the secretion of MMP-9 in MDA-MB 231 human breast cancer cells after 72 h, as assessed by zymography. One postulated mechanism by which IP6 prevents the cancer metastasis and inhibits MMPs may be due to its ability to chelate minerals such as iron and zinc (17, 27). All native MMPs contain catalytic domain with highly conserved zinc and calcium binding site. Zinc ion is necessary for enzyme activation, whereas calcium stabilizes enzyme structure (28). Dietary phytic acid can be quickly internalized by the cells and dephosphorylated to lower inositol phosphates, which further can act as secondary messengers and play an important role in regulation of signal transduction and many cellular processes (10, 29). The lower inositol phosphates do not reveal chelating property (30), therefore this mechanism seems questionable. We hypothesized that IP6 may affect matrix metalloproteinases expression indirectly via its action on transcriptional factors like AP-1 and NFkB, which regulate transcription of MMPs (3). Huang et al. (31) found that IP6 inhibits AP-1 activation. Our previously published study showed that IP6 influenced the expression of p65 subunit of nuclear factor κB and its IkBa inhibitor (32).
In conclusion, the results of this study show that IP6 modulates MMP-2, TIMP-1 and TIMP-2 genes expression in colon cancer cells at the transcriptional level in a way dependent on its concentration and time of interaction. The gene encoding MMP-9 was neither constitutively expressed nor induced by IP6 in Caco-2 cells.

REFERENCES