Polish Pharmaceutical Society

Colorectal cancer is the second leading cause of cancer incidence and death in Poland in the last years, claiming the lives of about 9,000 men and women a year (1). Molecular pathogenesis of colorectal cancer is still poorly understood. The observation, that patients with a chronic inflammation of the large bowel carry a much higher risk to develop colon cancer has implicated a role for the immune system as a tumor promoter in the colon (2). Epidemiological studies suggest that dietary phytochemicals may exert chemopreventive and therapeutic effect against cancer. Many studies reveal that the increased intake of selected bioactive food components such as fiber is negatively associated with etiology of some cancers, in particular colon cancer (3–5). Nevertheless, the specific molecular targets for the phytochemicals and the quantity needed to bring about the antineoplastic effects remain largely unresolved (6).

There is a growing interest in identifying new chemopreventive agents from dietary sources. Phytic acid, a hexaphosphorylated inositol (IP6) is an essential component of high fiber diet. It is physiologically present in the human large gut at concentrations reaching 4 mM (7). Recent studies have shown that IP6, both in vitro and in vivo, inhibits neoplastic growth of various types of cancer including colon carcinomas (4, 8). Despite anti-neoplastic effects available on IP6, the exact mechanisms underlying the cellular response to this compound are not fully understood and are still under investigation. IP6 has been shown to target cancer through multiple pathways, including modulation of cell signal transduction, inhibition of cell proliferation, and cell cycle progression, activation of apoptosis, induction of cell differentiation and anti-angiogenic potential (4, 8, 9). Recent studies indicated the ability of IP6 to modulate immune function of colonic epithelium through regulation of secretion of cytokines such as IL-6 or IL-8 (10).

Interleukin-6 (IL-6) is a multifunctional cytokine with pleiotropic effects. It contributes to a multitude of physiological and pathophysiological processes and exerts biological activities on various cell types including cancer cells (11). Recent data also suggest a potential role of this cytokine in colon cancer. IL-6 has been shown to promote the growth of some colorectal carcinoma cells in vitro, probably through a paracrine mechanism (12). IL-6 expression is low in normal, adenomatous and cancerous human colon mucosa, except in rather undifferentiated lesions, in which IL-6 is overexpressed (13). Clinical studies have shown that the levels of IL-6 are increased in the serum of patients suffering from colon carcinoma and IL-6 levels correlate with disease status (14, 15).

The aim of this study was to evaluate the effect of phytic acid on the expression of genes encoding IL-6 and its receptor IL-6R in human colorectal cancer cell line Caco-2.

EXPERIMENTAL

Cell culture

Human colon adenocarcinoma cell line Caco-2 was purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were grown under conditions as described elsewhere (16). Cells were treated with 1, 2.5 and 5 mM IP6 (pH 7.4) (Sigma Aldrich) for 1,
6, 12 and 24 h. The control Caco-2 cells were incubated under the same conditions without stimulation with IP6.

**RNA extraction**

Total RNA was extracted from control and IP6 treated cells with the use of TRIZOL® reagent (Invitrogen) following the protocol of the manufacturer. Cell lysis was carried out directly in a culture dish by adding 1 mL of TRIZOL reagent per 10 cm² of the area of the culture dish. RNA quality was assessed by 1.0% agarose gel electrophoresis in the presence of ethidium bromide. RNA concentration was determined spectrophotometrically using a GeneQuant pro (Amersham Biosciences).

**Real-time QRT-PCR assay**

Transcriptional activity of IL-6 and IL-6R genes was evaluated on the basis of copy number of mRNA related to 1 mg of total RNA by the use of real time QRT-PCR TaqMan technique. The analysis was carried out using an Opticon™ DNA Engine Continuous Fluorescence detector (MJ Research, USA). Gene expression profiles were determined using commercially available kits of oligonucleotide primers and probes specific for IL-6 (Hs 0017431_m1; Applied Biosystems) and IL-6R (Hs 00794121_m1; Applied Biosystems). Reaction mixture consisted of 25 µL 2× Quanti Tect Probe RT-PCR Master Mix, 0.5 µL QuantiTect RT Mix, 1× primers, probe mix and 0.1 µg RNA. 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 and at 60°C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 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). RT-PCR products were separated on 6% polyacrylamide gel (PAA) and visualized with silver staining.

**Statistical analysis**

Statistical analysis was performed with the use of Statistica PL 8.0 software. All the results were expressed as the means ± SD. The one-way ANOVA followed by Tukey’s test were applied to assess differences in the expression of examined genes between Caco-2 cell treated with IP6 and control cells. Significance was set at p < 0.05.

**RESULTS**

In the present study, mRNAs of IL-6 and its specific receptor IL-6R in colon cancer cells treated with phytic acid using real-time QRT-PCR assay were determined. In the first step of research, specificity of RT-PCR for the target genes was experimentally confirmed by PAA electrophoresis, which revealed the presence of single products of the predicted size (IL-6 – 95 bp; IL-6R – 118 bp) (Fig. 1).
The expression of IL-6 and IL-6R mRNAs was found both in the control cells and in all the cells treated with different doses of IP6 for 1, 6, 12, and 24 h. IL-6 mRNA was detectable at low level, i.e., $10^2$ copies per 1 mg of total RNA. IP6 at all doses for 1, 6, and 12 h had no effect on the transcriptional activity of gene encoding IL-6 (p > 0.05; ANOVA). A comparison of IL-6 transcript level in the control cells and cultures treated with different concentrations of IP6 for 24 h showed statistically significant differences (p = 0.0026; ANOVA). However, the transcriptional activity of IL-6 gene in unstimulated and stimulated cells with IP6 at the doses up to 2.5 mM was at the similar level. The expression of IL-6 mRNA was up-regulated in Caco-2 cells exposed to 5 mM IP6 in comparison with control (p = 0.01; Tukey test) (Fig. 2A).

At one hour, IL-6R gene was found to be expressed at the same level in both non-stimulated and IP6-stimulated cells (p = 0.1471; ANOVA).
Comparative analysis of IL-6R transcript level revealed statistically significantly different quantities in the control cells versus cultures exposed to IP6 for 6 h (p = 0.0009; ANOVA). There was no statistically significant difference between IL-6R mRNA expression in the control and cells treated with 1 and 2.5 mM IP6 for 6 h. The treatment of cells with the highest dose (5 mM) of IP6 for 6 h increased IL-6R mRNA level as compared to the control (p = 0.0017; Tukey test). A similar trend was observed after 12 and 24 h, where all IP6 doses caused significantly different expression of gene encoding IL-6R (p < 0.001; ANOVA). The amounts of IL-6R mRNA copies did not differ in the control cells and the cells treated with 1 mM IP6. Transcriptional activity of IL-6R gene was up-regulated in response to 2.5 mM IP6 after 12 h (p = 0.0254; Tukey test) and 24 h (p = 0.0002). The cells treated with 5 mM IP6 for 12 and 24 h showed a significant (p < 0.001; Tukey test) increase in the expression of IL-6R gene in comparison with control (Fig. 2A).

DISCUSSION AND CONCLUSION

Colon cancer is one of the most frequent and aggressive types of cancer and remains a significant global health concern (3). Inflammation is increasingly implicated in the etiology of colorectal cancer (2). IL-6 is a pleiotropic cytokine that can have both proinflammatory (11) and anti-inflammatory properties (17). There is evidence that IL-6 may exert anti-inflammatory and protective effects in intestinal mucosa and enterocytes (13, 18). Deregulation of IL-6 production is implicated in the pathology of some disease processes. Elevated IL-6 levels were observed in patients with chronic inflammation, as observed in colitis and Crohn disease (19). Interleukin-6 is one of the most ubiquitously deregulated cytokines in cancer, where it has been shown to play a role in growth stimulation, metastasis, and angiogenesis in several malignancies, including multiple myeloma, Kaposi’s sarcoma, glioblastoma, lung, renal, breast, ovarian and prostate cancer (20). The findings of recent investigations confirmed the role of IL-6 also in colorectal cancer (12). The expression of both IL-6 and IL-6R is increased in malignant tissue compared with normal colonic mucosa (21). Serum IL-6 concentrations are elevated in colorectal cancer patients compared with control group and correlate with the tumor size and histological grade (15, 22, 23). Current investigations have focused on the use of IL-6 as prognostic factor for cancer. Serum levels of IL-6 have been suggested to be a negative prognostic marker of survival in colorectal cancer (22). Moreover, IL-6 has been shown to promote the growth of colon cancer epithelial cells in a cell culture system in vitro (12).

The observations that the incidence of colorectal carcinoma was much greater in industrial societies compared with agricultural societies suggested that dietary factors might modulate the generation and progression of such tumor (5). In recent years, there has been growing interest in the activity of non-nutritional compounds present in the diet known as phytochemicals or nutraceuticals that improve overall health and/or reduce the risk of disease such as cancer (24, 25). Dietary interventions to attenuate colon carcinogenesis may be promising. An ideal chemopreventive agent should have little or no toxicity, high efficacy in multiple sites, capability of oral consumption, known mechanisms of action, low cost, and human acceptance (26).

Phytic acid, a natural dietary component, has revealed promising efficacy against various cancers but molecular mechanisms underlying this action remain unclear (4, 8). Previous study has shown that IP6 modulated immunologic function of the intestinal cell lines (10). Since IP6 shows pharmacological properties in the digestive system, their chemopreventive role needs investigation.

In the present study, we evaluated the influence of IP6 (1; 2.5 and 5 mM) on transcriptional activity of IL-6 and IL-6R genes in human colon Caco-2 cells. The Caco-2 cell line is frequently used to study enterocyte-related metabolic and inflammatory responses (27). Recent studies have shown that Caco-2 cells are an excellent model to study the regulation of cytokine expression and secretion by human enterocytes (28). The doses of IP6 used in the experiment corresponded to its concentrations in the lumen.

IL-6 and its receptor are expressed in colon cancer cells (21) and in human colon cancer cell lines, e.g., Caco-2, COGA, SW620, Colo201, LoVo and HT-29 (13, 29). Caco-2 colonocytes originate from a well differentiated tumor and exhibit high level of alkaline phosphatase – the differentiation marker (30). Vitkus and coworkers (28) have reported that Caco-2 cells secrete only very low levels of IL-6. Also Brozek et al. (30) have shown that IL-6 mRNA and protein concentrations were low in well and moderately differentiated Caco-2 cells, but in poorly differentiated COGA-13 cells the levels of this cytokine were high. Overexpression of IL-6 seems to be a hallmark of advanced tumor progression (30). In the present experiment, we also detected low concentrations IL-6 mRNA in Caco-2 cultures.
Phytic acid at concentrations up to 2.5 mM had no effect on the transcriptional activity of gene encoding IL-6. In response to 5 mM IP6 for 24 h the Caco-2 cells, however, expressed higher level of IL-6 mRNA. We have earlier shown that IP6 up-regulated constitutive secretion of IL-6 protein too (10). IL-6 exerts its effect via specific receptor on targets cells. It first binds to the IL-6R and next the complex IL-6/IL-6R associates with the membrane protein gp130 to generate intracellular signal transduction (31). Therefore, the evaluation of IL-6 expression has been considered in the context of transcriptional activity of gene encoding IL-6R. A possible autocrine loop in the intestinal epithelium can be supported by the results of this study showing that mRNA transcript for the receptor of IL-6 is also present in the intestinal Caco-2 cells. IP6 at all doses had no influence on alterations of IL-6R gene expression in Caco-2 cells for 1 h. The prolongation of cell incubation to 12 h and 24 h in the presence of IP6 at 2.5 mM evoked the increase in transcriptional activity of IL-6R gene. Cells treated with 5 mM IP6 at 6 – 24 h showed a significant increase in the expression of gene encoding IL-6 receptor.

In conclusion, IP6 at physiological concentration (1 mM) in the intestinal lumen failed to induce any alternations in IL-6 and IL-6R genes expression. The increase in IL-6 transcript level was evoked by 5 mM IP6 in the longest-lasting cultures. Along with the increasing IP6 doses and exposure time Caco-2 cells expressed successively higher IL-6R transcript level.

REFERENCES