

Expression of mRNAs of Urocortin in the STKM-1 Gastric Cancer Cell Line

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Abstract. *Background:* Urocortin is analogous to corticotrophin-releasing factors (CRFs) and a member of the CRF family. We previously demonstrated that urocortin mRNAs were expressed in both human and rat glioma cell lines, and that some of these lines transcribed the receptors. We hypothesize that urocortin might also be expressed in a gastric cancer cell line. The aim of the present study was to clarify the expression of mRNAs of urocortin1 (UCN1), -2 and -3 and of CRF and CRF receptors 1 and 2 in a gastric cancer cell line. *Materials and Methods:* STKM-1 a poorly-differentiated adenocarcinoma cell line was used. Transcripts in the cells were analyzed using cDNA. The fluctuation of mRNA with cellular stress, such as the one caused by a chemotherapeutic agent, serum supplementation and forskolin was examined. *Results:* Transcripts of UCN1, -2 and CRFR2 were expressed. No changes in transcription of UCN1 and UCN2 were observed with cellular stress. However, expression of CRFR2 mRNA transcripts significantly increased after an initial 24-h exposure to forskolin. *Conclusion:* Expression of the mRNAs of UCN1, 2 and CRFR2 was confirmed in the human gastric cancer cell line, STKM-1. Although the quantity of CRFR2 transcripts varied with forskolin, the overall transcription pattern was not influenced by cellular stimuli.

Corticotropin releasing factor (CRF) promotes the secretion of adrenocorticotrophic hormone (ACTH) in the pituitary gland and plays an important role in the stress response. Urocortin is analogous to CRF and a member of the CRF family (1, 2). Three types of urocortins, UCN1, UCN2 and UCN3, have been identified and these bind to CRF receptors.

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CRF receptors constitute a family of G protein-coupled receptors, and two major classes, CRF receptor type-1 (CRFR1) and type-2 (CRFR2), have been identified (3-5). CRFR1 binds with a higher affinity to CRF than urocortin (6). UCN1 has equal affinity for CRFR1 and CRFR2, while UCN2 and UCN3 specifically bind to CRFR2 (7).

In the central nervous system, urocortin mRNA expression is widespread (8). In particular, UCN1 is highly expressed in the Edinger-Westphal nucleus (2) and UCN3 is widely distributed in the hypothalamus and pituitary gland (9). UCN2 has been detected in the endometrium, myocardium, adrenal gland and peripheral blood cells. Urocortin responds via CRFR2 to stresses, such as anxiolysis, anorexia, vasodilation and myocardial contraction (10). In the gastrointestinal system, urocortin was found to be expressed in normal and inflammatory human gastric mucosa (11, 12). In carcinomas, urocortin was detected in adrenal cortical tumor, renal cell carcinoma, endometrial carcinoma, and prostatic carcinoma (13).

In a previous study, we demonstrated that urocortin mRNAs were expressed in glioma cell lines, and some of these lines transcribed the receptors (14). While normal and inflammatory human gastric mucosa and some malignant tumors express urocortin, whether or not mRNAs for urocortin and the receptors are expressed in a gastric cancer cell line is not known. We hypothesized that urocortin and the receptors might be expressed in a gastric cancer cell line. Accordingly, we examined the expression of urocortin mRNA in a gastric cancer cell line and studied the fluctuation of mRNA under cellular stresses, such as a chemotherapeutic agents, serum supplementation and forskolin.

Materials and Methods

Cells. STKM-1 is a human gastric cancer cell line established from malignant cells in the pleural effusion from a 41-year-old female patient. Histologically, the primary gastric cancer was revealed to be a poorly-differentiated adenocarcinoma. The cells cultured *in vitro* secrete carbohydrate antigen 19-9 (CA19-9) into the medium as a

Table I. Primers used for the study.

Gene	Sequence	
UCN1	5'CCCCGGGACAGACCCGTGTGTT	3'GGTTGTCCGGCGAGCGTCTGT
UCN2	5'ACCTGGACACCGCCACATGC	3'CCTGTGGGCCTCAGAGGGACT
UCN3	5'ACCCCGGAGCAGCCACAAGT	3'ACTTCCCTCCGCAGCGAAACG
CRF	3'CCGCCGAGGGCATTCTAGC	5'ACTTTTTCCGCGTGTGCTGC
CRFR1	5'AGGGCCTCTGGCTTCCTGC	3'TGGAGGGACATGCGTGCTGC
CRFR2	5'CAGACGGCCGCTGTGTGACC	3'ACGAGAGCCTGCCAGCACA

UCN: Urocortin, human; CRF: corticotropin releasing factor; CRFR: CRF receptor.

tumor marker (15). STKM-1 was cultivated in Roswell Park Memorial Institute (RPMI)-1640 medium with 10% fetal bovine serum (FBS) and grew as monolayers, with a doubling time of 31.4 h.

Treatments. The STKM-1 gastric cancer cells were dispersed and attached to the bottom of a culture flask 24 h before treatment. Therapies for gastric cancer, such as chemotherapy using 5-fluorouracil (5-FU), are in common clinical use. As cytotoxic stress, cells were exposed to 20 μ M of 5-FU at the 50% inhibitory concentration (IC_{50}) dose for 72 h. To investigate the effect of proliferative stimulation, cells were serum-starved for 72 h in medium with 0.25% FBS. After starvation, the cells were stimulated in RPMI-1640 medium, containing 10% FBS (Equitech-Bio, Kerrville, TX, USA). In a further experiment, cells were exposed to 10 μ M of forskolin, which increases intracellular cAMP. The cellular RNA was extracted by the acid guanidium-phenol-chloroform method (RNAzol B; Tel Test, Friendwoods, TX, USA) (16). Cells were also used for a cell-cycle analysis.

Reverse transcription-polymerase chain reaction (RT-PCR). The RNAs were treated with RNase inhibitor and RNase-free recombinant DNase I (Takara Bio, Ohtsu, Japan) for 30 min and the resulting total RNAs were reverse transcribed by Prime Script RT Master Mix (Takara Bio), then used for the polymerase chain reaction (PCR). Primers used for the study are shown in Table I. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (5'CCCAGCAAGAGCACAAGAG and 3'CCTCTCAAGGGGTCTACATG) was used as an internal control.

For quantification of the RNAs of urocortin and the *CRFRs*, quantitative PCRs were performed at three time points (incubation for 0 h, 12 h and 24 h) for each of the triplet samples by the ABI 7300 real-time PCR system at 95°C for 30 s followed by 40 cycles at 95°C for 5 s and then at 60°C for 31 s (CYBR Premix Ex Taq II; Takara Bio). Specificities of the reaction were confirmed with melting curves. When there were some non-specific patterns in the proliferating curve of real-time PCR, we selected the data with a simple peak pattern and analyzed them. The expression levels were compared by the relative quantification ($\Delta\Delta$ Ct) method (17). The products were also analyzed by 10% polyacrylamide gel and visualized by ethidium bromide.

Cell-cycle analysis. The cell cycle in STKM-1 was analyzed by a flow cytometer (FACScan; Becton Dickinson and Company, Franklin Lakes, NJ, USA) after propidium iodide (PI) staining (18). Cells (1×10^5) were dispersed with trypsin, suspended in phosphate-buffered saline (PBS), fixed with 75% ethanol, and stained with 6.6 μ M of PI with 180 units of RNaseA for 30 min.

The analyses were performed at three time points (incubation for 0 h, 12 h and 24 h). In the histogram, the horizontal axis shows fluorescence intensity and vertical axis shows cell counts.

Statistical analysis. Statistical analysis was performed by two-sample *t*-test.

Results

When the transcription of urocortin was examined in the human gastric cancer cell line, STKM-1, the RNAs were transcribed, as shown in Figure 1. Transcripts of *UCN1* and -2, and *CRFR2* were expressed, whereas that of *UCN3*, *CRF* and *CRFR1* was not detected. RT-PCR for *GAPDH* showed amplification of a 130 bp-band, confirming RNA quality. Amplification of *UCN1*, -2 and *CRFR2* revealed the expected size of 100 bp.

Since we predicted that urocortin transcription would fluctuate under cellular stress, we evaluated the effects of cytotoxic conditions, cell proliferation, and the addition of forskolin on the transcription of these molecules. When STKM-1 cells were exposed to 5-FU for 24 h, expression of *UCN1* mRNA transcripts decreased ($p=0.54$). Expression of *UCN1* and *CRFR2* mRNA transcripts changed little over time (Figure 2). When STKM-1 cells were stimulated by serum supplementation, expression of *UCN1*, 2 and *CRFR2* mRNA transcripts showed no significant change for the initial 24 h. After 24 h had passed, expression of each was slightly increased (not significantly) (Figure 3). When STKM-1 cells were cultivated with forskolin, expression of *CRFR2* mRNA transcripts was significantly increased after the initial 24 h ($p=0.006$). The quantities of the transcripts were maintained at 48 h. However, expression of *UCN1* and -2 mRNA transcripts had little change over time (Figure 4).

The impact of 5-FU on the cell-cycle distribution was examined. As time of 5-FU exposure elapsed, the proportion of cells in S, G_2 and M phases decreased as seen by flow cytometry (Figure 2). After serum supplementation, the G_0/G_1 and S phase proportions increased as seen by flow cytometry (Figure 3). The addition of forskolin led to a slight increase in the G_2 and M phase proportion (Figure 4).

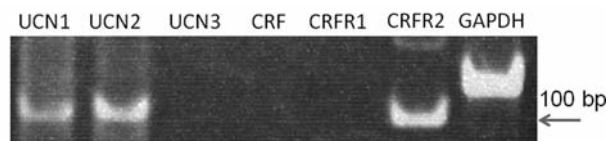


Figure 1. Expression of mRNA of urocortin-1 (*UCN1*), *UCN2*, *UCN3*, corticotrophin-releasing factor (*CRF*), *CRF* receptor-1 (*CRFR1*) and *CRFR2* in the human gastric cancer cell line *STKM-1*. After extraction of RNA, transcripts were treated with *DNase I*, cDNAs were synthesized and then amplified by a thermal cycler (product size, 100 bp). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control (121 bp).

Discussion

In the present study, we investigated the expression of *UCN1* and -2, and *CRFR1* and -2 in the *STKM-1* gastric cancer cell line. Although fluctuations in transcription were observed, the overall transcription pattern was not influenced by exposure of the cells to a chemotherapeutic agent, serum supplementation, or forskolin.

In a previous study, the expression of mRNAs of *UCN1*, -2, -3, and *CRF* and *CRFR1* and -2 was examined in five malignant glioma cell lines. The RNAs of *UCN1* and -2 were transcribed in all examined human glioma cell lines. Four out of five human glioma cell lines transcribed *UCN3*. Additionally, some cell lines transcribed *CRFR1*, and others transcribed *CRFR2*. The effect of proliferative and cytotoxic stimulation by serum supplementation, ionizing radiation, and the anti-neoplastic agent temozolomide were investigated. The transcription pattern was not influenced by these stimuli in that study either (14). In the present study, *UCN3* and *CRFR1* were not detected in the *STKM-1* gastric cancer cell line, unlike in glioma cell lines. Since *CRFR1* is expressed in central tissues and *CRFR2* is expressed in peripheral tissues, the difference between the results of these two studies might be due to differences in the genetic tissue, which would cause a corresponding difference in the expression of *CRFR2* in the gastric cancer cell line. *UCN2* was detected in *STKM-1* cells similarly to glioma cell lines. Generally, *UCN1* and -3 are distributed widely in various human tissues, but *UCN2* is expressed poorly, except for endometrial and gestational tissues (19). Accordingly, this result might be noteworthy, similarly to the glioma cell lines.

In the present study, forskolin significantly increased the *CRFR2* mRNA level, although that of *UCN1* and -2 did not increase. Kageyama *et al.* revealed that raised intracellular cAMP levels induced by forskolin increased the *UCN1* mRNA level in endothelial cells of the human umbilical vein (20). They reported that the *UCN1* mRNA levels fell transiently to 67% of the control value within two hours of addition of 10 μ M forskolin, followed by an increase to

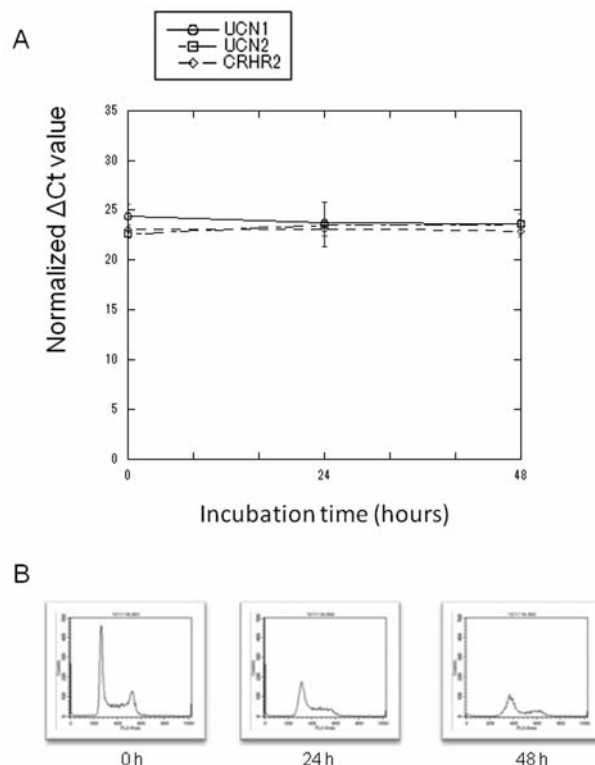


Figure 2. A: Effect of 5-fluorouracil (5-FU) on the expression of transcripts of urocortin (*UCN*)-1, *UCN2* and *CRFR2*. Cells were exposed to 20 μ M of 5-FU at the 50% inhibitory concentration (*IC50*). *UCN1*, *UCN2* and *CRFR2* mRNAs in the *STKM-1* cell line at three time points (0 h, 24 h, 48 h) were evaluated by quantitative polymerase chain reaction (PCR). Values are the means of experiments conducted in triplicate, bars=S.D. B: Effect of 5-fluorouracil (5-FU) for *STKM-1* was detected by flow cytometry. The analyses were performed at three time points (0 h, 24 h, 48 h). Horizontal axis shows the fluorescence intensity and vertical axis shows cell counts.

224% of the control level within six hours. The *UCN2* mRNA levels also fell transiently to 51% of the control value within two hours of addition of 10 μ M forskolin in a dose-dependent manner (21). In this study, forskolin might have influenced the transcription of urocortins within 24 h and then induced an increase in transcription of *CRFR2*.

Some studies have reported on urocortin in stomach and in gastric cancer. Chatzaki *et al.* detected the expression of *UCN1*, and *CRFR2* in normal human stomach (11) and also reported that *CRF*, *UCN1* and *UCN2* reduced the degree of apoptosis of gastric cancer cells, which were transiently transfected to express functional *CRFR2*, whereas this effect was not detected in non-transfected cells (12). This finding suggests that *CRFR2* had a protective biological role. Therefore, an antagonist to *CRFR2* might induce apoptosis of a gastric cancer cell line expressing *CRFR2*. Cheng *et al.* conducted an immunohistochemical analysis of urocortin in

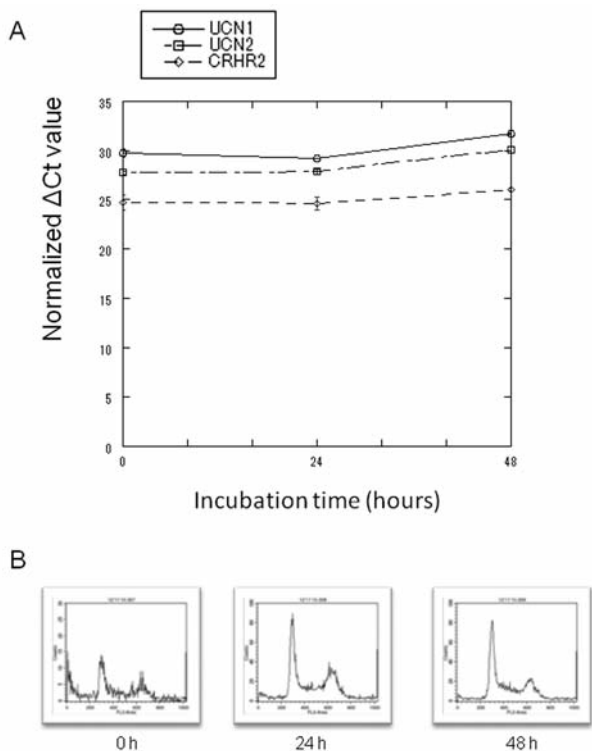


Figure 3. A: Effect of serum supplementation on the expression of transcripts of urocortin (UCN)-1, UCN2 and CRFR2. Cells were serum-starved for 72 h in medium with 0.25% Fetal bovine serum (FBS) and then stimulated in RPMI-1640 medium, containing 10% FBS. UCN1, UCN2 and CRFR2 mRNAs in the STKM-1 cell line at three time points (0 h, 24 h, 48 h) were evaluated by quantitative polymerase chain reaction (PCR). Values are the means of experiments conducted in triplicate, bars=S.D. B: Effect of serum supplementation for STKM-1 was detected by flow cytometry. The analyses were performed at three time points (0 h, 24 h, 48 h). Horizontal axis shows fluorescence intensity and vertical axis shows cell counts.

gastric cancer (22). The immunoscore for urocortin was high in the gastric mucosa and significantly higher in well-differentiated and moderately-differentiated gastric adenocarcinoma than in poorly-differentiated tumors. Moreover, urocortin expression was higher in gastric adenocarcinomas with neuroendocrine differentiation than in mucinous adenocarcinomas and signet-ring cell carcinomas. Consequently, UCN expression inversely correlates with a higher tumor grade and advanced TNM stage in gastric adenocarcinomas. In the present study, although STKM-1 is a poorly-differentiated tumor cell line without neuroendocrine differentiation, mRNA expression of UCN1 and -2 was detected. If a more highly differentiated gastric cancer cell line than STKM-1 were to be used, quantification of mRNA of urocortin might be more substantial.

The present study has certain limitations. It is a fact that the role of urocortin is not entirely clear, several studies on

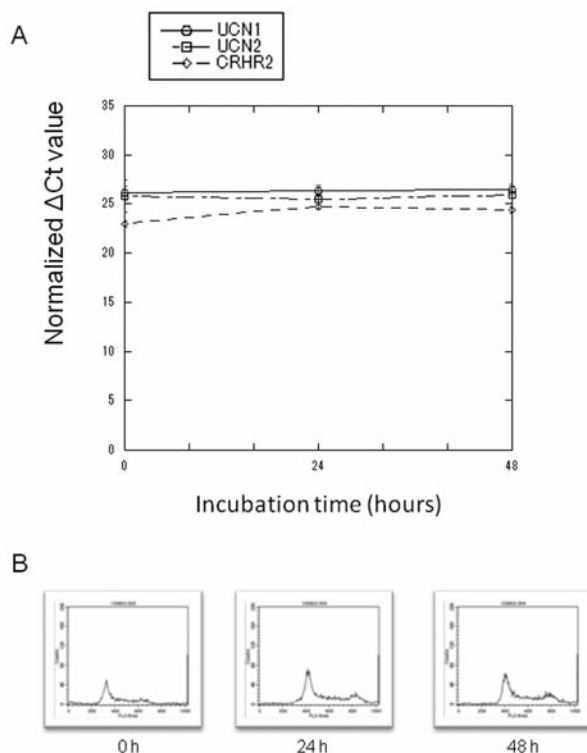


Figure 4. A: Effect of forskolin on the expression of transcripts of urocortin (UCN)-1, UCN2 and CRFR2. Cells were exposed to 10 μ M of forskolin, which increases intracellular cAMP. UCN1, UCN2 and CRFR2 mRNAs in the STKM-1 cell line at three time points (0 h, 24 h, 48 h) were evaluated by quantitative polymerase chain reaction (PCR). Values are the means of experiments conducted in triplicate, bars=S.D. B: Effect of forskolin for STKM-1 was detected by flow cytometry. The analyses were performed at three time points (0 h, 24 h, 48 h). Horizontal axis shows fluorescence intensity and vertical axis shows cell counts.

the role of urocortin via CRFR2 for cancer cells have been reported. Bale *et al.* showed that activation of CRFR2 induced tonic suppression of angiogenesis and remodeling of the vasculature, and that the level of vascular endothelial growth factor decreased in rat smooth muscle cells by urocortin treatment via CRFR2 (23). Hao *et al.* demonstrated that the CRFR2 ligand, UCN2, can suppress the growth of Lewis lung carcinoma cell tumors *in vivo* and *in vitro* predominantly by inhibiting tumor vascularization, but also potentially through direct effects on tumor cell proliferation (24). Wang *et al.* showed that urocortin inhibited hepatocellular carcinoma angiogenesis *in vitro* and reduced tumor microvessel density *in vivo* via CRFR2 (25). Furthermore, they also found that urocortin was able to directly inhibit the growth of human small cell lung carcinoma cells. It was also shown that the activation of CRFR2 could inhibit p38 and protein kinase B

phosphorylation to suppress the secretion of vascular endothelial growth factor in small cell lung cancer cells (26). Taking all of these results into consideration, we predict that urocortin and *CRFR2* are involved in the growth of tumor cells and that *CRFR2* may be a therapeutic target in cancer.

There have also been some reports about the impact of urocortin on the function of the stomach. When *UCN1* was administered centrally or peripherally, it induced impaired gastric emptying, reduced plasma ghrelin and reduced gastric acid secretion (27, 28). *UCN2* also had the same effects leading to a reduction in food intake, which was inhibited with *CRFR2* antagonist (29). These findings might be related to anorexia induced by cachexia in patients with gastric cancer. Additionally, *UCN1* is increased in gastric inflammation by *Helicobacter pylori* and is increased much more after pylorus eradication. Moreover, the anti-inflammatory effect of *UCN1* on the gastric mucosa was also considered a possible factor (11). These findings might be related to carcinogenesis induced by inflammation.

In conclusion, expression of the mRNAs of *UCN1* and -2, and *CRFR2* was confirmed in the STKM-1 human gastric cancer cell line. Although the quantity of *CRFR2* transcripts varied with exposure to forskolin, the overall transcription pattern was not influenced by cellular stimuli. Further study is warranted to elucidate the roles of *UCN1* and -2 and their receptors.

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