Expression of mRNAs of Urocortin and Corticotropin-releasing Factor Receptors in Malignant Glioma Cell Lines

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Abstract. *Background: Urocortin and corticotropin-releasing factors (CRFs) and their receptors are expressed in many organs, including the central nervous system. In this study, the expression of mRNAs of urocortin 1, 2, 3, and CRF and CRF receptors 1 and 2 in malignant glioma, was examined. Materials and Methods: The RNAs of human and rat glioma cell lines were isolated. Transcripts in these cells were analyzed using cDNA. In addition, the effects of proliferative and cytotoxic stimulation by serum supplementation, ionizing radiation, and the antineoplastic agent temozolomide were investigated. Results: Human and rat cells transcribed urocortin. CRF receptors were detected in human glioma cells. When human KNS42 cells were exposed to stimulation, transcription was altered according to the specific condition. Conclusion: Expression of mRNAs of urocortin and CRF receptors was confirmed in human glioma cell lines. Although the quantities of transcripts varied with the proliferative and cytotoxic stimulation, the overall transcription pattern was not influenced by these stimuli.*

Urocortin is a member of the sauvagine/corticotropin-releasing factor (CRF)/urotensin I family and promotes the secretion of pro-opiomelanocortin (POMC)-derived peptides, adreno-corticotropic hormone (ACTH), beta-endorphin, and melanocyte-stimulating hormone in the pituitary gland (1-2). Three types of urocortin, urocortin-1, urocortin-2, urocortin-3, have been identified and both CRF and urocortin bind to CRF receptors. CRF receptors constitute a family of G protein-coupled receptors, and two major classes, CRFR1 and CRFR2, are recognized (3-5). CRFR1 binds with a higher affinity to CRF than urocortin. In contrast CRFR2 demonstrates a higher affinity to urocortin and this difference suggests that they are endogenous ligands of the respective peptides.

In the central nervous system, urocortin has effects on stress and appetite. While the properties of urocortin mimic the effects of CRF, such as enhancement of anxiety and activity, urocortin suppresses appetite and feeding behavior (6). More precisely, central injection of urocortin-3 modulates feeding (7), blood glucose levels, and hypothalamic POMC gene expression (8). It has been suggested that CRFR2 is the main mechanism for this (9).

In the normal brain, *urocortin* mRNA expression is widespread (10). Urocortin-like immunoreactivity is also detected in every region of the brain, including the hypothalamus, pons, cerebral cortex, and cerebellum. Urocortin is known to be distributed in regions other than the brain and has been investigated in the cardiovascular system (11) and other organs. Immunoreactivity and mRNA have been detected in the human placenta, spinal cord, lymphocytes, and ovary (12-15). In the rat, urocortin expression has been detected in the thymus, spleen, gastrointestinal tract, testis, kidney, and liver (16). Expression of urocortin and CRFRs has been studied in prostate carcinoma (17) and the signaling role of receptors in the prostate has been demonstrated (18).

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Key Words: Urocortin, corticotropin-releasing factor receptors, glioma.

Table I. *Primers used for the study.*

Human(h) urocortin (UCN) 1	5' CCCCGGGACAGACCCTGTGTT	3' GGTTGTCGGCGAGCGTCTGT
hUCN2	5' ACCTGGACACCGCCACATGC	3' CCTGTGGGCCTCAGAGGGACT
hUCN3	5' ACCCCGGAGCAGCCACAAGT	3' ACTTCCCTCCGCAGCGAAACG
hCRF	5' ACTTTTTCCGCGTGTTGCTGC	3' CCGCCGAGGGCATTCCTAGC
hCRFR1	5' AGGGCCTCTGGCTTCCCTGC	3' TGGAGGGACATGCGTGCTGC
hCRFR2	5' CAGACGGCCGCTGTGTGACC	3' ACGAGAGCCTGCCCAGCACA
rat(r) UCN1	5' CCCGGAGAGCAGCCAGTGGA	3' ACCTCCGCCCTGATTCCGCA
rUCN2	5' CCCATTGGCCTCCTGCGGAT	3' GGCGGCCAACACGGGCTAGT
rUCN3	5' CCGGGGCACCAAGTTCACTCT	3' CGCTGCCTTGGCTCGCAAAT
rCRF	5' CCCCAGCAACCTCAGCCGAT	3' AGTTGGGGGACAGCCGAGCA
rCRFR1	5' TGAGGTCCGCTCCGCTATCCG	3' CGGGGAGGTGGGGATGGACA
rCRFR2	5' GCTCCGCCCTGAGAAAGCGG	3' ATCCTGGTGGGCGATGTGGGA

When our group performed a comprehensive analysis of transcripts in glioma, we found that the mRNAs of urocortin and its receptors were expressed in the glioma. As for intracranial tumors, urocortin is known to be expressed in pituitary adenomas, such as growth hormone-producing adenoma and non-functioning adenoma (19). However, these tumors are benign, unlike most gliomas, and strictly speaking, not of central nervous system origin. To confirm the transcription of urocortin mRNA and its receptors, five human and three rat glioma cell lines were examined here. Each type of urocortin and CRFR, as well as the effect of serum supplementation, ionizing radiation and chemotherapeutic agent on one human glioma line was investigated.

Materials and Methods

Cells. Human KNS42 (20), T98G (21), A172, U138MG, and U373MG (ATCC, Rockville, MD, USA) glioma cells were cultivated in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS). Rat-9L (23-25), C6 (ATCC), and RT2 (26) were also cultivated in the same medium.

Treatments. The KNS42 glioma cells were dispersed and attached to the bottom of a culture flask 24 h before treatment. Cells were exposed to a therapeutic dose of ionizing irradiation, 2 Gy, generated by an MBR-1520R instrument (Hitachi, Tokyo, Japan), or 583.4 μM of temozolomide, provided by Merck & Co., Inc. (NJ, USA), at the 50% Inhibitory Concentration (IC_{50}) dose after 72 h treatment (27). In another experiment, cells were serum-starved for 72 h in medium with 0.5% FBS. After starvation, the cells were stimulated by medium, containing 10% FBS (Equitech-Bio, Kerrville, TX, USA). The cellular RNA was extracted by the acid guanidium-phenol-chloroform (AGPC) method (RNAzol B; Tel Test Inc., Friendswoods, TX, USA) (28). Cells were also used for 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 PrimeScript RT Master Mix (Takara Bio), then used for the polymerase chain reaction (PCR). Primers used for the study were shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5' CCCAGCAAGAGCACAAGAG and 3' CCTCTTCAAGGGGTCTACATG, was used as an internal control. The PCR was carried out at 98˚C for 30 s followed by 40 cycles at 98˚C for 10 s, at 60˚C for 10 s and then at 72˚C for 60 s (Phusion High-Fidelity DNA Polymerase, Finnzymes; Thermo Scientific, Vantaa, Finland). After extension for 5 min at 72˚C, the products were analyzed by 10% polyacrylamide gel and visualized by ethidium bromide.

For quantification of the RNAs of urocortin and the CRFRs, quantitative PCRs were performed 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 34 s (CYBR Premix Ex Taq II; Takara Bio). Specificities of the reaction were confirmed with melting curves. The expression level was compared by the relative quantification (ΔΔ Ct) method (29).

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

Statistical analysis. Statistical analysis was performed by Student's *t*-test.

Results

When the transcription of urocortin was examined in human cell lines, glioma cells transcribed the RNAs as shown in Figure 1. By using additional rat cell lines, it was demonstrated that the phenomenon was not restricted to humans. The RNAs of urocortin-1 and -2 were transcribed in all the examined human glioma cell lines. Four out of five human cell lines transcribed urocortin 3. In the rat, urocortin-2 was detected in all the cell lines. However, not all the cell lines transcribed urocortin-1 and the -3 mRNAs. Urocortin-1 and -3 were not detected in the C6 cell line. CRF was not detected except in the human A172 cell line. Four human cell lines, U138MG, KNS42, A172 and T98G, transcribed CRFR1. Transcription of CRFR2 was also observed in the U138MG,

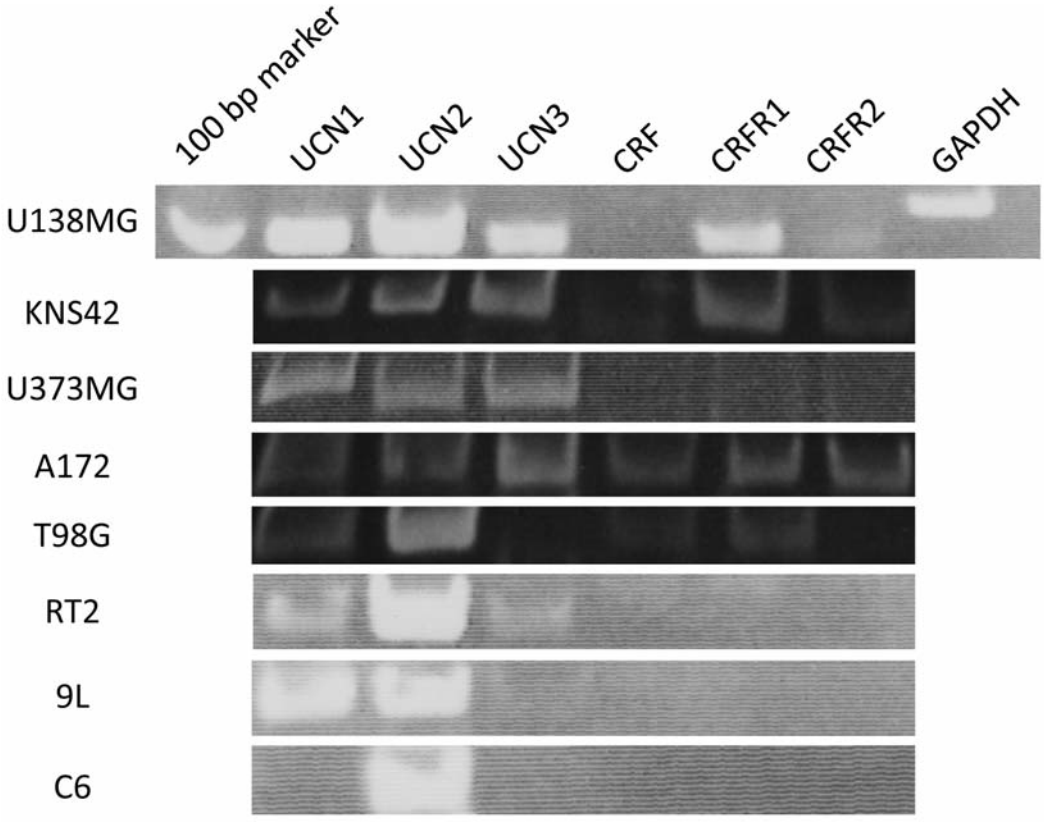

Figure 1. *Expression of mRNA of urocortin-1, urocortin-2, urocortin-3, corticotropin-releasing factor (CRF), corticotropin-releasing factor receptor-1 (CRFR1) and CRFR2 in human and rat glioma cell lines. After extraction of RNA, transcripts of urocortin-1 (UNC1), UNC2, UCN3, CRF, CRFR1 and CRFR2 were treated with DNAse I, cDNAs synthesized and then amplified by a thermal cycler (products size, 100 bp). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control (121 bp).*

KNS42, and A172 cell lines. In contrast, transcription of these receptors was not detected in rat cell lines.

Since urocortin and the receptors were transcribed in human glioma cell lines, we tried to evaluate the effects of cellular stresses including cell density, proliferation and cytotoxic conditions on transcription of these molecules. In regard to cell density, KNS42 cells have a strong tendency to attach to each other, thereby increasing cell density (22). In addition, KNS42 cells enter the quiescent cell phase when they become confluent. Based on these phenomena, different confluent phases of the cells were analyzed in this cell line (Figure 2). Transcription of urocortin 2 decreased, although only slightly. No other significant changes were observed.

In spite of the difference in cell confluency, stress derived from the cell density did not appear to confer any effect on the transcription of urocortin and the CRFRs. Therefore, to investigate the effect of proliferative stimulation, after serum deprivation, cells were cultured under serum-supplemented conditions. When the cells were stimulated by 10% FBS, transcription of urocortin-2 and -3 significantly increased at 24 h (Figure 3). However, no further increase was observed at 48 h, and the increase was transient. No changes were observed in the other urocortins and receptors.

Meanwhile, therapies for malignant glioma, such as irradiation and chemotherapy using nitrosourea, especially temozolomide, have been in common clinical use. The effects of such interventions were analyzed. One dose of 2 Gy, frequently used for fractionated therapy with ionizing radiation, did not result in any change in the transcription of urocortin or the receptors (Figure 4). Although the cell-cycle distribution changed, only a slight down-regulation of urocortin-3 was observed at 48 h after irradiation. The effect of temozolomide was also trivial and the agent did not have any impact on the transcripts (Figure 5).

Discussion

Urocortin is a 40-amino-acid peptide cloned from the Edinger Westphal nucleus of the midbrain (2). Urocortin-1 analogs urocortin-2 and urocortin-3 have been identified by human

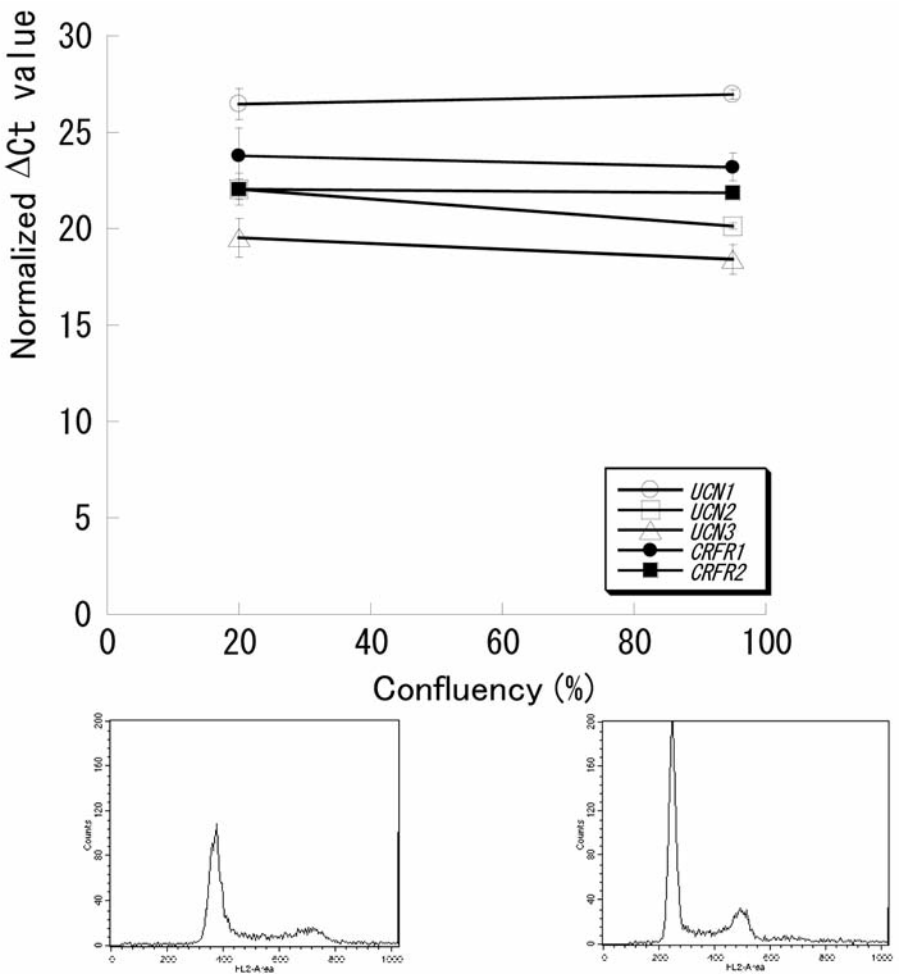

Figure 2. *Effect of confluency on the expression of transcripts of urocortin and receptors. Urocortin-1 (UCN1), UCN2, UCN3, corticotropin-releasing factor receptor-1 (CRFR1) and CRFR2 mRNAs in the KNS42 cell line at different cell confluencies (20% and 95%) were evaluated by quantitative PCR. Normalized ΔCt was calculated as 40-(respective gene Ct-GAPDH Ct). The values are the means of experiments conducted in triplicate, bars=S.D.*

genome database searches (31, 32). Urocortin-1 and urocortin-3 are expressed in various human tissues, however, urocortin-2 is strongly expressed in the endometrium and gestational tissues (33). The current data demonstrated that urocortin-2 was expressed in all human and rat glioma cell lines tested. This result might be noteworthy, because to our knowledge, this distribution has not been recognized until the present study, and while both urocortin-1 and -2 suppress feeding behavior in the hypothalamic region, the signals were assumed to be different. Unlike urocortin-1, urocortin-2 was found to potently suppress feeding *via* a CRFR2-dependent mechanism without eliciting malaise (34). Loss of feeding behavior is one of the problems in the treatment of tumor-bearing patients. This problem needs to be re-addressed further from the standpoint of urocortin-2 in patients with glioma. Moreover, while all the cells originated from glial tissue, transcription of CRF was not detected in any, except in the A172 cell line. We examined the tramscription of CRF on the human neuroblastoma SK-N-MC cell line (35). The result demonstrated that CRF expression was detected, accompanied by that of urocortin-1, urocortin-2, and CRFR1 (data not demonstrated). However, transcription of CRF was not universal in tumors of glio-neural origin. Additional investigation will be required for the determination of CRF distribution in glio-neural malignancies.

Variability of urocortin other than in the neuro-endocrine system has been studied in inflammatory states. Urocortin-1 increased under inflammatory conditions, such as ulcerative colitis (36, 37) and rheumatoid arthritis (38). Other roles in peripheral tissue have been studied mostly in the cardiovascular system. Urocortin, but not CRF, was expressed in cardiac myocytes and expression of urocortin mRNA was

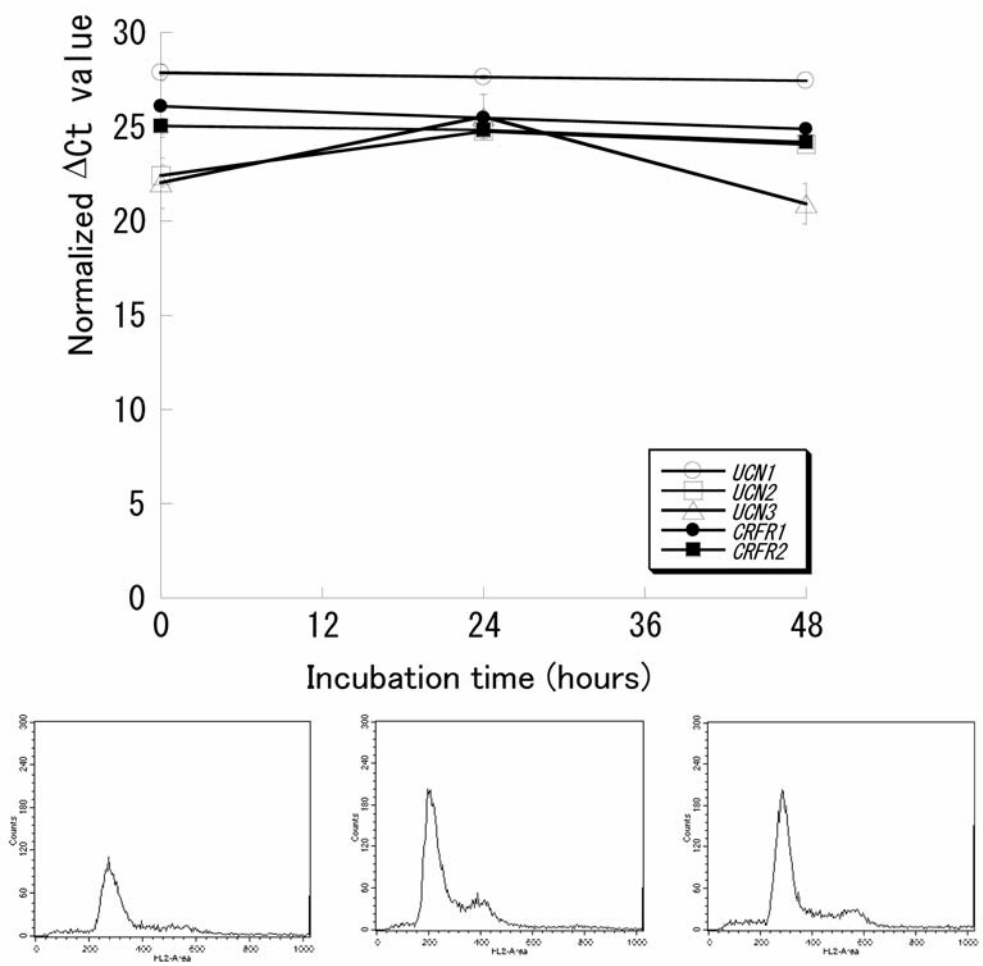

Figure 3. *Effect of serum supplementation on the expression of transcripts of urocortin and receptors. After 72 h of serum starvation with 0.5% fetal bovine serum (FBS), cells were stimulated in culture medium with 10% FBS supplementation. Urocortin-1 (UCN1), UCN2, UCN3, corticotropin-releasing factor receptor-1 (CRFR1) and CRFR2 mRNAs in the KNS42 cell line at each time point were evaluated by quantitative PCR. Values are the means of experiments conducted in triplicate, bars=S.D.*

increased 12–18 h after thermal injury (11). Stimulation of CRFR2 by CRF and urocortin-1 induced the release of atrial and brain natriuretic peptides (39, 40). Urocortin had a positive inotropic action on the heart (41), led to an increase in both protein and DNA synthesis in cardiac fibroblasts (40, 42) and demonstrated a cardioprotective action against hypoxia (43). The protective effect was also observed in models of Parkinson's disease (44). Moreover, urocortin-1 was found to exert an antioxidative action in response to the angiotensin II-induced generation of reactive oxygen species by endothelial cells (45). In these cases, CRFR2, which might be an endogenous receptor for urocortin, was assumed to be responsible for the action (46, 47).

Signal pathways of CRFRs are now better-understood. Cardioprotective action against ischemia/reperfusion injury was found to be involved in CRFR1-mediated extracellular signal-regulated protein kinase-1 and -2 (ERK1/2) signaling pathways (48). In addition, pathways of protein kinase A (PKA) (40, 49, 50), phosphoinositide-3 kinase (PI3-K) (51), p38 mitogen-activated kinases (MAPKs) (50, 52), protein kinase C (PKC) (53), protein kinase B/AKT (51), tyrosine-protein kinase Src (SRC) (48), cyclooxygenase 2 (COX-2) (52), and endothelial nitric oxide synthase (eNOS) (54) have been reported. For instance, hypertrophy-inducing action of urocortin, manifested by increases in the secretion of atrial and brain natriuretic peptides from cardiomyocytes and an increase in protein synthesis may be induced by the PKA and AKT pathways (40, 49, 55), while the cardioprotective action of urocortin against hypoxia and reperfusion injury may involve SRC, ERK1/2, PKC, and PI3-K (48, 51). In peripheral organs, such pathways have not been well-clarified other than in the cardiovascular system. In

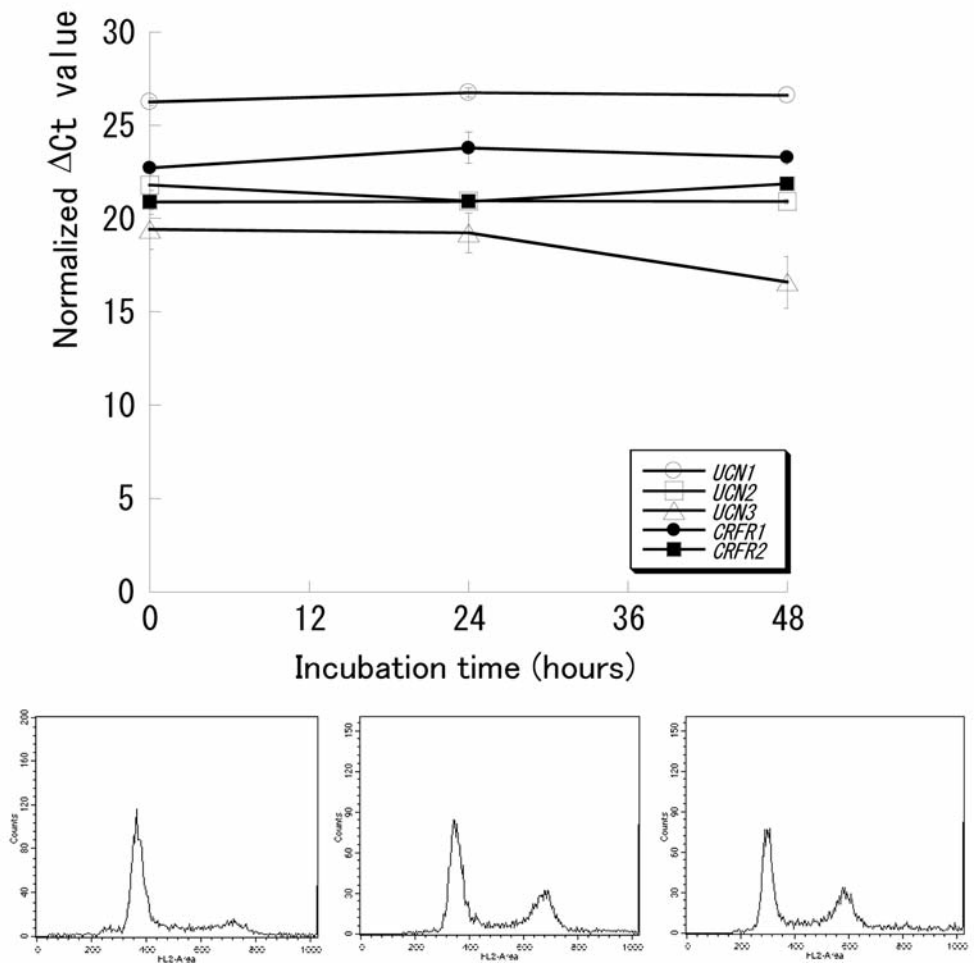

Figure 4. *Effect of ionizing radiation on the expression of transcripts of urocortin and receptors. Cells were exposed to 2 Gy of ionizing radiation. Urocortin-1 (UCN1), UCN2, UCN3, corticotropin-releasing factor receptor-1 (CRFR1) and CRFR2 mRNAs in the KNS42 cell line at each time point were evaluated by quantitative PCR. The condition corresponds to the clinical setting of the dose used for fractionated therapy with ionizing radiation. Values are the means of experiments conducted in triplicate, bars=S.D.*

malignancies, the level of urocortin expression remains controversial. Both mRNA and peptide expression decreased in endometrial adenocarcinoma (56). Both non-neoplastic and prostate adenocarcinoma expressed urocortin and in non-neoplastic tissues, urocortin was localized in the secretory luminal epithelial and basal layer cells, in the smooth muscle component of the stroma, and in lymphoid infiltrates. Intense immunostaining was evident in prostate adenocarcinoma cells (17). An *in vitro* study demonstrated that both CRFR1 and CRFR2 were expressed in a mouse prostate carcinoma cell line and CRF was reported to promote apoptosis, whereas urocortin-2 exerted the opposite effect. CRF reduced BCL-2 expression, induced BCL-2-associated X protein (BAX) expression, and hyperpolarized the mitochondrial membrane potential to activate caspase-9. On the contrary, urocortin-2 increased BCL-2 and reduced BAX expression, in which phosphorylation of AKT and cyclic AMP response element-binding was involved (57). In humans, benign prostate tissue and prostate carcinoma specimens differentially expressed CRFR2. Furthermore, urocortin expression in prostate carcinoma has been shown to be identical to non-malignant prostate tissues, but expression loss of CRFR2 in prostate carcinoma was hypothesized to contribute to prostate tumorigenesis, progression, and neoangiogenesis (18).

Little is currently known in regard to the role of these proteins in gliomas. The current study demonstrated that *urocortin* mRNAs were expressed in both human and rat glioma cell lines, and some of these lines transcribed the receptors. Although fluctuations of such transcription were

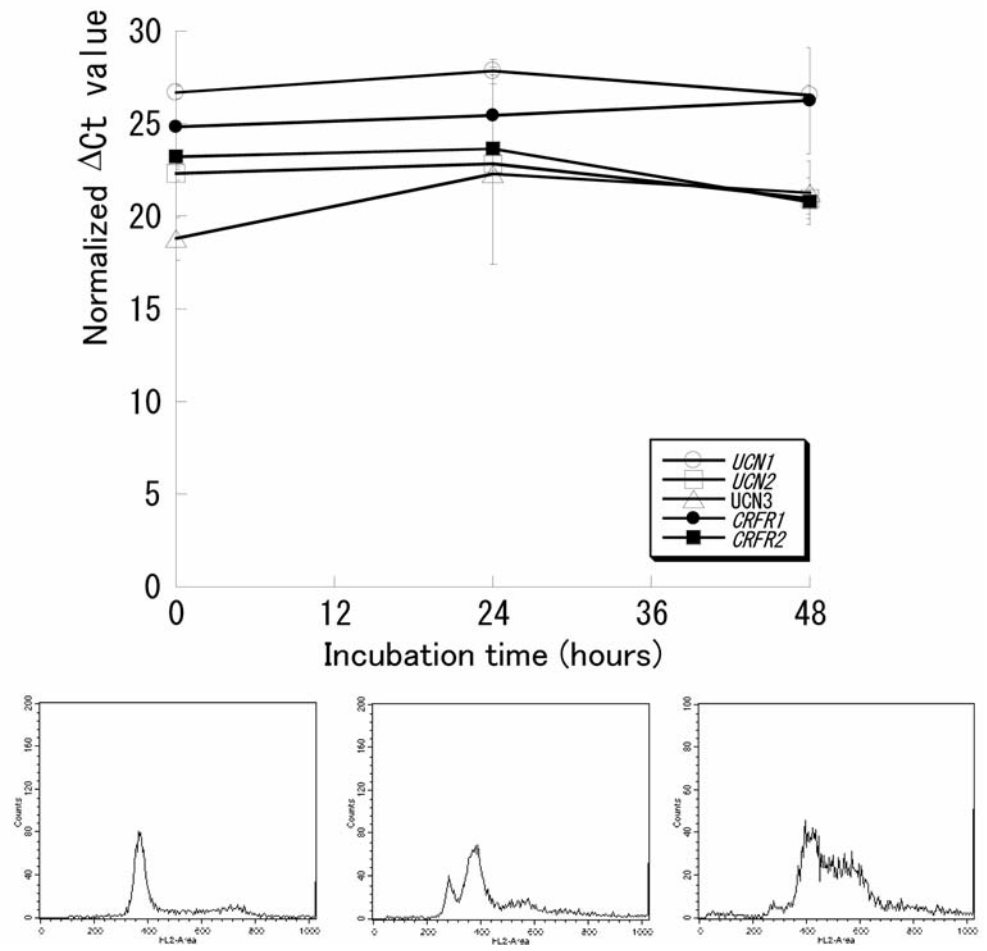

Figure 5. *Effect of temozolomide on the expression of transcripts of urocortin and receptors. Cells were exposed to 583.4 μM of temozolomide. The condition corresponds to 50% inhibitory concentration (IC_{50}) of temozolomide after 72 h of treatment. Urocortin-1 (UCN1), UCN2, UCN3, corticotropin-releasing factor receptor-1 (CRFR1) and CRFR2 mRNAs in the KNS42 cell line at each time point were evaluated by quantitative PCR. Values are the means of experiments conducted in triplicate, bars=S.D.*

observed, the overall transcription pattern was not influenced by serum supplementation, ionizing radiation, or representative chemotherapeutic agents. In order to further understand the functions of urocortin in gliomas, experiments including the administration of each peptide as well as forced-expression of receptors need to be addressed. Growing numbers of splice-variant isoforms of CRFR1 and CRFR2 have been identified (58). The expression and roles of such isoforms need to be explored. Further study is warranted.

Acknowledgements

This research was supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Scientific Research (C). The Authors acknowledge Keiko Tomaru and Mayumi Nomura of the Jikei University School of Medicine for their expert technical assistance.

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Received September 24, 2012
Revised October 26, 2012
Accepted October 29, 2012