Biochemical and Biophysical Research Communications 457 (2015) 706–711

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The inhibitory effect of hypoxic cytotoxin on the expansion of cancer stem cells in ovarian cancer

Noriko Nozawa-Suzuki [a], Hideko Nagasawa [b], Ken Ohnishi [c], Ken-ichirou Morishige [a, *]

[a] Department of Obstetrics and Gynecology, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan
[b] Laboratory of Pharmaceutical and Medical Chemistry, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1195, Japan
[c] Department of Biology, Center for Humanity and Sciences, Ibaraki Prefectural University of Health Sciences, Inashiki-gun, Ibaraki 300-0394, Japan

ARTICLE INFO

Article history:
Received 22 December 2014
Available online 22 January 2015

Keywords:
Hypoxia
HIF (hypoxia inducible factor)
Cancer stem cell
Stem cell factors

ABSTRACT

While an increase in progression free survival time is seen when an angiogenesis inhibitor is used in the treatment of high-relapse rate ovarian cancer, it has little effect on overall survival. A possible cause of treatment-resistance to angiogenesis inhibitors is the growth of stem cells in a hypoxic microenvironment built inside the tumor tissue by angiogenesis inhibition. In this study, we examined the possible suppression of stem cell and cancer stem cell (CSC) expansion by hypoxic cytotoxin, TX-402.

TX-402, an analogue of tirapazamine, has been developed as a hypoxia selective prodrug with inhibitory effects of HIF-1 and angiogenesis. We considered TX-402 as a possible molecular-target drug candidate for ovarian cancer due to its inhibition of CSC expansion. In this study, we found that the expressions of HIF-1α and HIF-2α were increased under hypoxia in serous ovarian cancer cell lines. The expressions of HIF-1α and HIF-2α induced under hypoxia were repressed by TX-402 in a dose-dependent manner. Next, we investigated the effects of hypoxia on the expression levels of stem cell factors, Oct4, Nanog, Sox2 and Lin28, and showed that their expressions were induced by hypoxia. It was also observed that the expressions of putative ovarian cancer stem cell markers, CD133 and CD44 were induced under hypoxia. Furthermore, TX-402 was found to dose-dependently inhibit the expressions of CSC markers and stem cell factors.

Oct4 expression was repressed by HIF-2α silencing, but not by HIF-1α silencing, indicating that TX-402 may repress the expression of Oct4 by inhibiting HIF-2α.

We constructed CaOV3 spheroids as a 3-dimensional hypoxia model, in which the internal hypoxic region contained CSC-like cells expressing Oct4. The internal hypoxic region, which contained Oct4 expressing cells, disappeared following TX-402 treatment.

In conclusion, hypoxia promoted the expansion of CSCs expressing CD133 and CD44 accompanied by an increase of stem cell factors. Its inhibition of hypoxia-induced CSC expansion makes TX-402 promising agent usable in combination for ovarian cancer therapy.

1. Introduction

Ovarian cancer is the leading cause of deaths in gynecologic malignancies. Due to the asymptomatic nature of the disease, most women are diagnosed when the disease has progressed to an advanced metastatic stage. Currently, the standard chemotherapy has achieved an initial response rate of 70–80%. However, even in patients who initially respond well, most patients relapse within a relatively short period of time [1]. The acquisition of chemoresistance in recurrent tumors is one of the greatest obstacles to achieving a complete cure. It has been shown that recurrent ovarian cancer includes cancer stem cells (CSCs) which express stem cell markers such as ALDH1 and CD133 are chemoresistant, suggesting that CSCs may be responsible for recurrence [2]. In several hematologic and solid tumors, CSCs are known to be the most tumorigenic and treatment-resistant of all heterogeneous tumor cells [3,4].

Many solid tumors, including those of ovarian cancer, possess poorly vascularized regions that are severely hypoxic. It has recently been found that hypoxia plays a direct role in maintaining the cancer stem cell population [5] under the control of

* Corresponding author. Department of Obstetrics and Gynecology, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan.
E-mail address: mken@gifu-u.ac.jp (K.-i. Morishige).

http://dx.doi.org/10.1016/j.bbrc.2015.01.053
0006-291X/

overexpressed hypoxia inducible factor (HIF). HIFα subunits, which contain paralogs such as HIF-1α, HIF-2α, and HIF-3α, are stabilized under hypoxia and can form heterodimers with β subunits, and then bind to hypoxia response element (HRE) in target gene promoters [6]. In contrast, under normoxia, HIFα undergoes prolyl hydroxylation, binds to a ubiquitin E3 ligase (Von Hippel-Lindau protein), thereby leading to polyubiquitination-dependent rapid proteasomal degradation. It has been reported that both HIF-1α and HIF-2α are critical to cancer stem cell maintenance in glioma [7,8].

Emerging data shows that CSCs have similar characteristics to normal stem cells [9]. Recently, Takahashi and colleagues found that the four stem cell factors (Oct4, Klf4, Sox2, C-Myc) could reprogram human cells into an undifferentiated state [10]. Takahashi and colleagues also showed that the induction of iPS generation by hypoxia was more effective because hypoxia induced the expression of these stem cell factors [11,12]. Thus, we first examined the expression of HIF-1α, HIF-2α and the stem cell factors under hypoxic conditions in ovarian cancer.

A hypoxic cytotoxin, 3-Amino-2-quinoxalinecarbonitrile 1,4-dioxide (TX-402), which is an improved analog of tirapazamine (TPZ), has been shown to inhibit HIF-1α expression [13]. We therefore hypothesized that TX-402 might improve the prognosis of malignant ovarian cancer by inhibiting HIF and CSC expansion. Here, we demonstrated that the expressions of HIFs and mRNA levels of stem cell factors were induced under hypoxia in ovarian cancer cell lines, and evaluated that the possible use of TX-402 as a molecular targeted drug due to its dose-dependent reduction of the induction of these factors under hypoxia.

2. Materials and methods

2.1. Cell culture

Serous ovarian cancer cell lines, CaOV3 and SKOV3ip1 (provided by the Gynecological and Obstetrics Department, Osaka University) were cultured in Dulbecco's modified medium (Wako, Osaka, Japan) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Wako) under 5% CO_2. Hypoxic conditions were set in a Modular Incubator Chamber MIC-101 (Billups-Rothenberg, Del Mar, CA) by flushing with a gas mixture containing 1% O_2, 5% N_2.

2.2. Western blotting

30 μg protein was analyzed by SDS-PAGE and transferred to Hybond C nitrocellulose (GE Healthcare, Tokyo, Japan). Proteins were detected using primary antibody, anti-HIF-1α (Santa Cruz Biotechnology, Dallas, TX), anti-HIF-2α (Novus Biologicals, Littleton, CO) and anti-β-actin (AnaSpec, San Jose, CA). Horseradish peroxidase conjugated secondary antibodies (Promega, Madison, WI) and ECL Plus blotting detection reagents (GE Healthcare) were used according to the manufacturer's instructions.

2.3. Real time PCR

mRNA levels were analyzed using a One Step SYBR PrimerScript RT-PCR Kit (Takara Bio, Ohtsu, Japan). The following primers were used (Takara Bio):

Oct4: forward 5′-TGAAGCTGGAGAAGGAGAACTG
reverse 5′-GCAGATGGTCGTTTGGCTGA-3′
Sox2: forward 5′-GTGAGCGCCCTGCAGTACAA-3′
reverse 5′-GCGAGTAGGACATGCTGTAGGTG-3′
Lin28: forward 5′-GAAGTCAGCCAAGGGTCTGGAA-3′
reverse 5′-GGCATGATGATCTAGACCTCCACA-3′
Nanog: forward 5′-CCTGTGATTTGTGGGCCTGA-3
reverse 5′-CTCTGCAGAAGTGGGTTGTTTG-3′
CD133:forward5′-CCAAGGACAAGGCGTTCACA-3′
reverse5′-TGTTGCCATGGACTTAATCTCATCA-3′
CD44:forward 5′-AGTCTTCACAAACCCTTGA-3′
reverse 5′-CTTCCAGAGTTACGCCCTTGAG-3′
HIF-1α: forward 5′-CAGCCGCTGGAGACACAATC-3′
reverse 5′-TTTCAGCGGTGGGTAATGGA-3′
HIF-2α: forward 5′-CATGCGCTAGACTCCGAGAACA-3′
reverse 5′-GCTTTGCGAGCATCCGGTA-3′.

2.4. Small interfering RNA

Small interfering RNA (siRNA) targeting HIF-1α, HIF-2α and random sequence siRNA was used as control (Takara Bio):

HIF-1α siRNA: 5′-CCA CAU UCA CGU AUA UGA UTT-3′
HIF-2α siRNA: 5′-GGA CAU AGU AUC UUU GAC UTT-3′
random control RNA: 5′-UCU UAA UCG CGU AUA AGG CTT-3′

Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.5. Spheroid generation and culture

Two thousand cells/well in sphere medium were distributed on PrimeSurface plates (Sumitomo Bakelite, Tokyo, Japan). Plates were incubated in an atmosphere of 5% CO_2 at 37 °C for 10 days.

The sizes of 16 spheroids were measured under a light microscope.

2.6. Immunofluorescence on frozen section

Spheroids were fixed for 2 h with 10% formalin containing 10% sucrose, washed with phosphate buffered saline, and then embedded in O.C.T. compound (Sakura FineTek, Tokyo, Japan) for the creation of frozen sections. Sections were permealized with 0.5% TritonX-100 and incubated with antibodies against OCT4 (C-10, sc-5279: Santa Cruz Biotechnology) for 1 h and DAPI (Dojindo, Kumamoto, Japan) for 20 min.

Hypoxia detection was performed with a Hypoxyprobe-1 Plus Kit (Hypoxyprobe, Burlington, MA). Pimonidazole hydrochloride (Hypoxyprobe-1) was added to the culture medium and incubated for 2 h. The frozen sections were incubated with FITC conjugated monoclonal antibody (Hypoxyprobe).

2.7. Statistical analysis

All experiments were conducted with a minimum of three samples. The statistical significance of differences between groups was calculated by Student's two-tailed t test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Hypoxia induces the expression of HIF-1α in ovarian cancer cell lines

We examined the induction of HIF-1α expression under hypoxia in ovarian serous cancer cell lines, CaOV3 and SKOV3ip1 by western blotting of the cell extracts taken at indicated intervals during incubation under 20% and 1% oxygen. As shown in Fig. 1A, HIF-1α expression was induced in both cell lines after 2 h under hypoxic conditions.

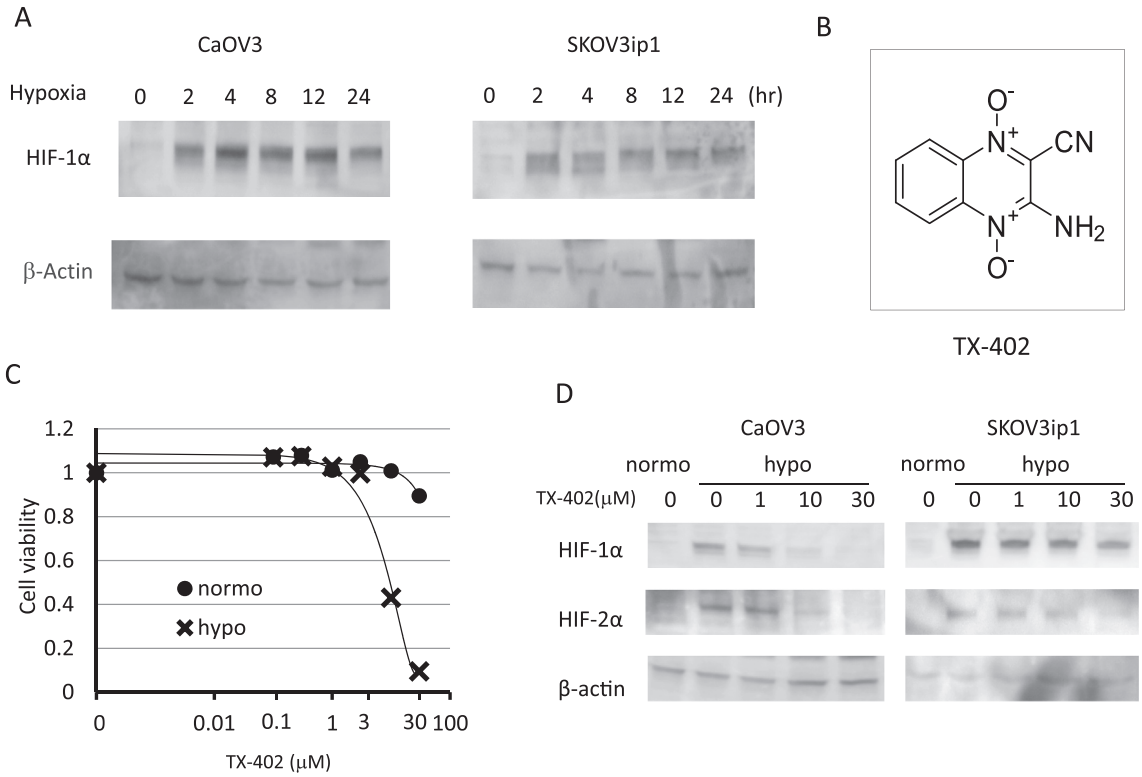

Fig. 1. Hypoxia-induced expression of HIF-1α and HIF-2α and the effect of TX-402 on their expressions. A. Expression of HIF-1α under hypoxia. The cell lysates of CaOV3 and SKOV3ip1 under normoxia (20% O_2) and hypoxic conditions (1% O_2) for the indicated time were subjected to immunoblotting using antibodies against HIF-1α. B. The structural formula of TX-402 C. Cytotoxicity of TX-402. WST-assay was performed after 48 h culture under normoxia and hypoxia with indicated concentration of TX-402. IC_{50} is 8.78 μM D. Effect of TX-402 in HIF-1α and HIF-2α expression. CaOV3 and SKOV3ip1 were exposed to hypoxia for 48 h with TX-402 for indicated time. HIF-1α and HIF-2α, β-actin protein levels were detected by western blotting.

3.2. TX-402, a hypoxic cytotoxin represses HIF expression under hypoxia

The chemical structure of TX-402, a hypoxic cytotoxin is shown in Fig. 1B. A WST-1 assay was performed to examine the cytotoxicity of TX-402 to CaOV3 cells under normoxia and hypoxia for 48 h in the presence and absence of TX-402 (Fig. 1C). TX-402 showed selective cytotoxicity under hypoxia, with an IC_{50} of 8.78 μM.

Next, we examined the effect of TX-402 on the expression of HIF-1α and HIF-2α. As shown in Fig. 1D, TX-402 repressed the expression of HIF-1α and HIF-2α that was induced under hypoxia in a dose-dependent manner.

3.3. Inhibitory effects of TX-402 on expressions of stem cell factors and CSC markers under hypoxia

It has recently been reported that hypoxia induces stem cell character [10]. We therefore examined whether hypoxia induces the expression of stem cell factors, such as Oct4, Sox2, Nanog and Lin28, and the effects of TX-402 on the mRNA levels of these stem cell factors. As shown in Fig. 2A, mRNAs were expressed at significantly higher levels after 48 h of hypoxia treatment than under normoxia, indicating that hypoxia may induce stemness. Next, we examined the effect of TX-402 on the induced expression of these stem cell factors under hypoxia and found that TX-402 clearly reduced the expressions of these factors in a dose-dependent manner. Furthermore, we examined the expressions of CD133 and CD44, which are known as putative ovarian cancer stem cell markers [14,15], under hypoxia and the effect of TX-402 on their expressions. The hypoxia-induced expression of these markers was also significantly repressed by TX-402 in a dose-dependent manner (Fig. 2B).

3.4. HIF-2α regulates Oct4 expression under hypoxia

To determine whether HIF-1α or HIF-2α is involved in the induction of stem cell factors, we examined the mRNA expression of stem cell markers by silencing HIF-1α or HIF-2α with their respective siRNAs. As shown in Fig. 3A and B, the administration of siRNAs against HIF-1α and HIF-2α induced a marked repression of HIF-1α and HIF-2α mRNAs, respectively. The siRNA against HIF-2α but HIF-1α significantly decrease the expression of Oct4. A modest, but not significant, decrease in Sox2 expression was induced by the si RNA of HIF-2α.

Neither HIF-1α nor HIF-2α silencing had any effect on the expressions of Nanog or Lin28 induced under hypoxia. We also examined the effects of HIF-1α and HIF-2α siRNAs on the expressions of CD133 and CD44, but no statistically significant effects were observed (data not shown). Thus, hypoxia might promote the expansion of CSCs through HIF-2α accompanied with the increase of stem cell factors.

3.5. TX-402 effect in spheroid model

We constructed a 3-dimensional spheroid model of CaOV3 cells, which mimicked the characteristics of solid tumors with hypoxic regions [16]. An aliquot of 2×10^3 cells/well was incubated on a PrimeSurface 96-well plate (diameter: 500 μm) for 10 days. The size of 16 spheroids was measured under a light microscope. Their average size is shown in Fig. 4A. The size of spheroids treated with

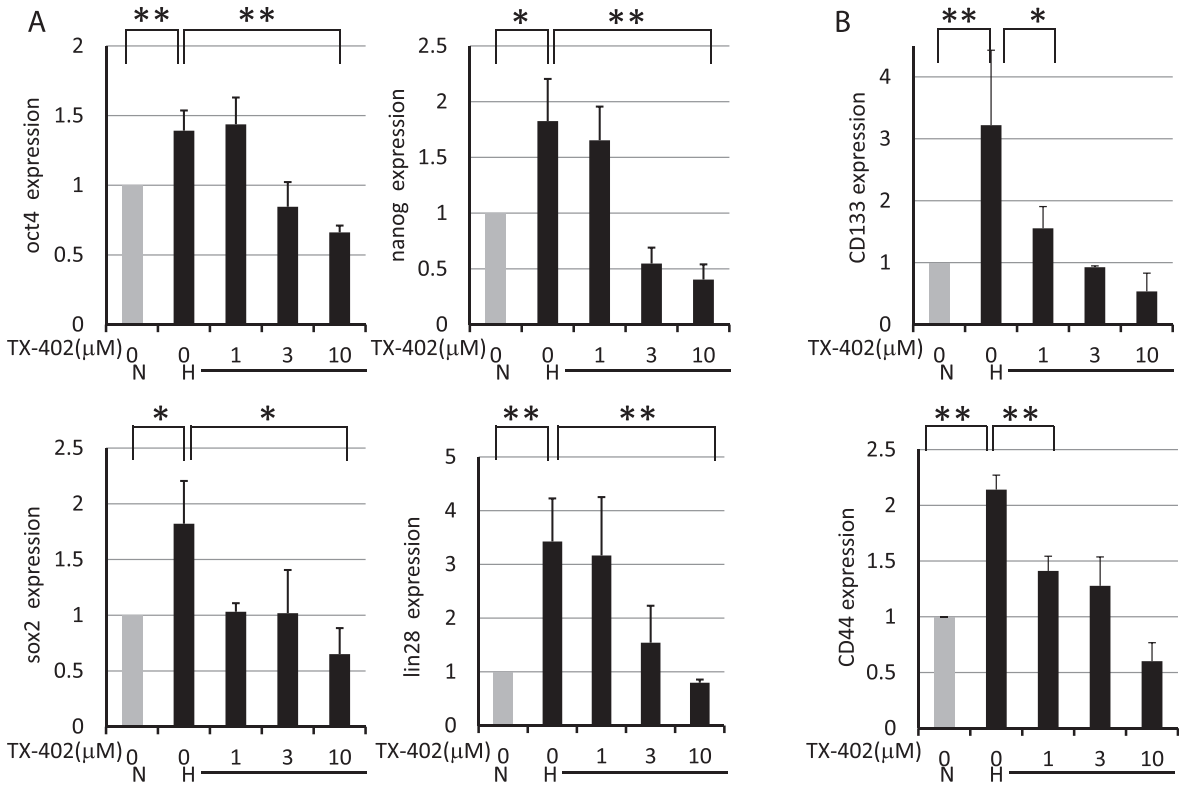

Fig. 2. Effect of TX-402 on the expression of A) stem cell factors; Oct4, Nanog, Sox2, Lin28 and B) putative ovarian cancer stem cell markers, CD133 and CD44. CaOV3 cells were cultured for 48 h under normoxic (20% O_2) or hypoxic (1% O_2) conditions. The total RNA of CaOV3 was extracted and mRNA levels of Oct4, Nanog, Sox2 and Lin28 were analyzed by real time PCR. N: normoxia, H: hypoxia $^{*}P < 0.05$, $^{**}P < 0.01$.

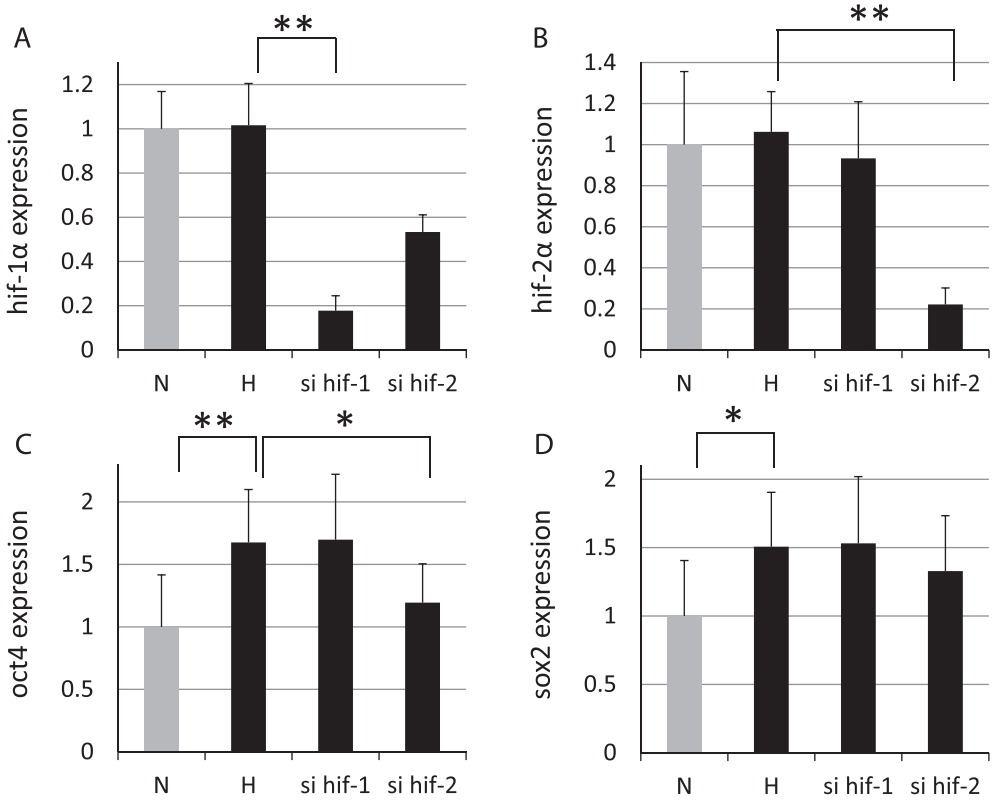

Fig. 3. Effect of HIF-1α and HIF-2α silencing on the expression of Oct4 and Sox2. siRNAs against HIF-1α and HIF-2α were transfected into CaOV3 cells. The transfected cells were incubated under normoxic or hypoxia for 48 h mRNA levels of HIF-1α, HIF-2α, Oct4 and Sox2 were analyzed by real time PCR. Results are shown as means ± SEM of three experiments performed in triplicate. N: normoxia, H: hypoxia $^{*}P < 0.05$, $^{**}P < 0.01$.

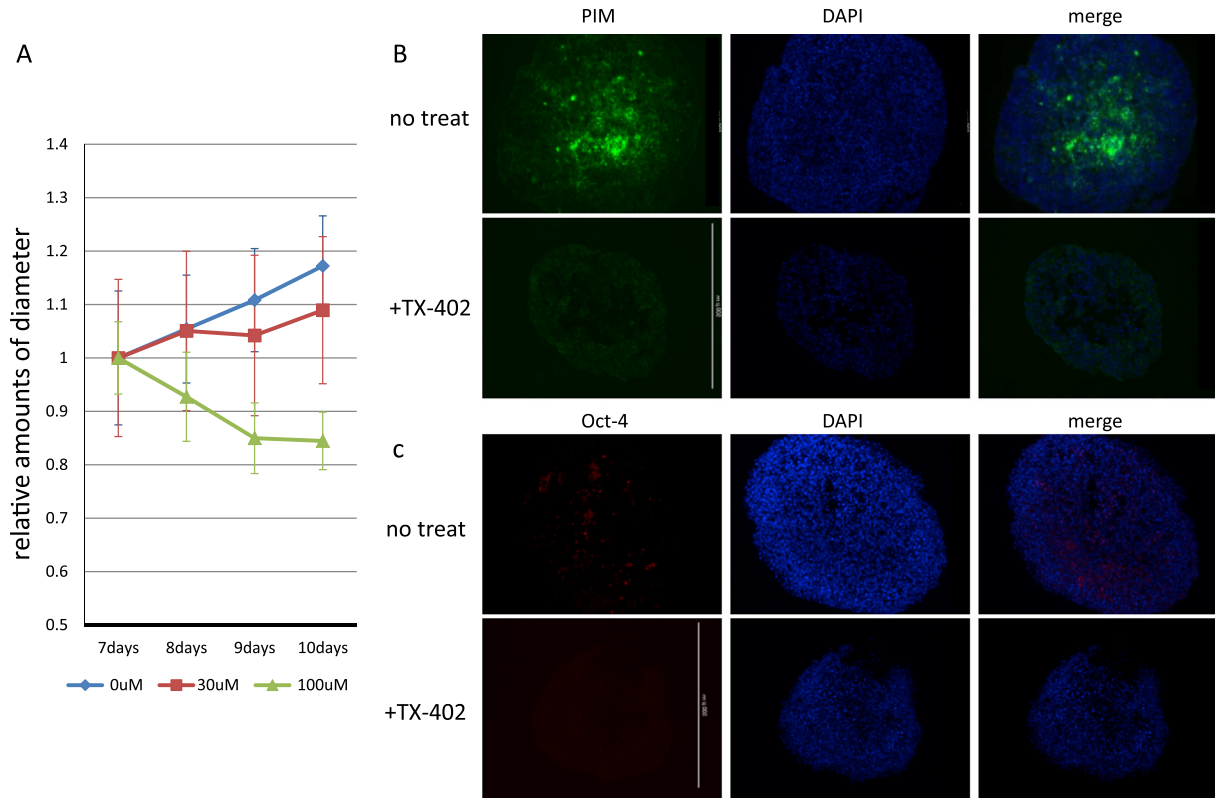

Fig. 4. 3D spheroid model: A) the size of 16 spheroids was measured and the relative amounts against the size on day7 are shown. B, C) Immunofluorescence of spheroid model B. left: pimonidazole, middle:DAPI, right:merge, upper:no treatment, lower:100 mM of TX-402 for 24 h C. left:Oct-4, middle:DAPI, right:merge, upper:no treatment, lower:100 mM of TX-402 for 24 h.

TX-402 decreased dose- and time-dependently. The hypoxic regions of the frozen sections of the spheroids were assessed by immunofluorescence using hypoxia marker pimonidazol (Fig. 4B and C). The existence of hypoxia was indicated if the central area of spheroid was positive for the marker. The disappearance of pimonidazol-positive hypoxic area after 24 h of treatment with TX-402 suggests that TX-402, a hypoxic cytotoxin, selectively killed the cells in the hypoxic region, which led to a decrease in the size of the spheroids (Fig. 4A). Furthermore, a few cells expressing Oct4 were detected inside the spheroids. That the expression of Oct4 disappeared following treatment with TX-402, suggests that the inner hypoxic cells killed by TX-402 included the stem-like cells expressing Oct4.

4. Discussion

Although approximately 70% of ovarian cancer patients respond to the primary therapy, most eventually relapse with chemoresisitant recurrence [1]. Recently, CSCs have been revealed to play a key role in recurrence and chemoresistance, suggesting CSC as a potential target of cancer treatment [2,3]. It has been demonstrated that hypoxia is the required microenvironment to produce CSCs in solid tumors [5]. Under hypoxia, HIF is overexpressed and considered to be the key regulator in controlling over 100 genes to survive under hypoxia [6]. In this study, we examined whether the hypoxic cytotoxin, TX-402 (Fig. 1B), inhibits the expansion of CSCs through reducing HIF expression. In serous ovarian cancer cell lines, CaOV3 and SKOV3ip1, HIF-1α expression was clearly induced under hypoxia (Fig. 1A). As shown in Fig. 1C, TX-402 showed the specific cytotoxicity under hypoxia. Since TX-402 was known to inhibit the expression of HIF-1α, we examined whether TX-402 repressed the expression of HIF-1α and HIF-2α in ovarian cancer cells (CaOV3, SKOV3ip1). As we expected, the expressions of both were dose-dependently inhibited by TX-402 (Fig. 1D).

It is well known that the stem cell factors, Oct4, Sox2 and Lin28 produce iPS cells [10]. Recently, it has been observed that a hypoxic microenvironment was suited for stem cell growth, because of the hypoxia-induced expression of the stem cell factors [12]. In ovarian cancer CaOV3 cells, these factors were also induced under hypoxia and their expressions were found to be inhibited by TX-402 (Fig. 2A).

CD133 and CD44 are considered to be putative ovarian cancer stem cell markers [14,15]. We found that the mRNA levels of these markers increased under hypoxia in CaOV3 cells and that their inductions were inhibited by TX-402 in a dose-dependent manner (Fig. 2B). Although we also examined the effects of hypoxia on ALDH1, which is well known as a putative ovarian cancer stem cell marker [16–18], it was not induced under hypoxia (data not shown).

As TX-402 inhibited the HIFs expressions, we examined the effects of silencing HIF-1α and HIF-2α using their siRNAs, on the expression of stem cell factors, to determine which is relevant for the inhibition of stem cell factors. The hypoxia-induced expressions of Oct4 and Sox2 were found to be repressed by the siRNA of HIF-2α (Fig. 3). The mRNA levels of other stem cell factors (Nanog and Lin28) and cancer stem cell markers (CD133 and CD44) were repressed by neither HIF-1α nor HIF-2α silencing (data not shown). Oct4 is essential for maintaining an undifferentiated cell fate in embryonic stem cell [19] and it was reported that HIF-2α binds to the Oct4 promoter and induced its expression [20]. Thus, HIF-2α might be implicated in the regulation of the induction of Oct4 expression under hypoxia in CaOV3 cells. As for the other stem cell

markers, there no significant effects were observed with either of the siRNAs, however TX-402 resulted in a significant reduction. Accordingly, there may be some unknown mechanism, other than the inhibition of HIFs, for CSC marker expression by TX-402.

Solid tumors are heterogeneous and contain a poorly vascularized hypoxic area. A 3D tumor spheroid is an appropriate model for mimicking intratumoral microenvironments, such as hypoxia [21,22]. We constructed a 3D spheroid as a hypoxia model for CaOV3. Spheroids were successfully formed after 10 day culture of 2×10^3 cells per well on a PrimeSurface plate. The average diameter of the 16 spheroids treated with TX-402 decreased significantly both dose- and time-dependently. Immunofluorescence staining of their frozen sections was performed to detect hypoxic regions and Oct4 expression in the spheroids. Fig. 4A (left) shows the hypoxic region inside the spheroids. After TX-402 was added to the culture medium for 24 h, the inner hypoxic region disappeared (Fig. 4A, right). As for Oct4, a similar result is shown in Fig. 4B. The inner hypoxic cells including Oct4-expressing cells disappeared following TX-402 pretreatment (Fig. 4B, right). There may be two possibilities, the first is that the inner hypoxic cells, including CSCs, were killed with TX-402. The second is that the population that highly expressed stem cell factors and putative cancer stem cell markers was selectively killed. Further experiments will be necessary to elucidate the precise mechanism by which TX-402 killed the CSCs in the in hypoxic region. The results obtained in this study suggest that TX-402 might be useful for hypoxia-targeted therapy to inhibit hypoxia-induced CSC expansion in ovarian cancer and that it might release therapeutic resistance to angiogenesis inhibitors.

Acknowledgments

Serous ovarian cancer cell lines, CaOV3 and SKOV3ip1 were kindly gifted from the Gynecological and Obstetrics Department, Osaka University. We thank Ms Tomoyo Katabe and Ms Yukie Ikeshita (Laboratory of Pharmaceutical and Medical Chemistry, Gifu Pharmaceutical University) for their technical supports. This work was supported by MEXT KAKENHI Grant Number 25462588 and Grant for Clinical Research Promotion from Gifu University and Gifu Pharmaceutical University.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.053.

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