Original article

GABA-receptor agonist, propofol inhibits invasion of colon carcinoma cells

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ABSTRACT

Propofol (2,6-diisopropylphenol), one of the most commonly used intravenous anaesthetic agents during cancer resection surgery, has been reported to have the ability of influencing the invasion of human cancer cells. In the present study, using the human colon carcinoma cell line LOVO, we demonstrated that propofol stimulation significantly decreased the expression of MMP-2 and -9 and subsequently decreased the invasive activity of the cancer cells. Because MAPK signaling is one of the key regulators of MMP expression, we further evaluated MAPK signaling after stimulation with propofol. It was found that propofol stimulation inhibited the phosphorylation of MAPKs, including ERK1/2, JNK, and p38. Deactivation of ERK1/2 phosphorylation was sustained for up to 12 h, while deactivation of phosphorylation of JNK and p38 returned to the endogenous level by 30 min. It was noteworthy that the ras/raf/MEK/ERK pathway inhibitor PD98059 attenuated the down-regulation of propofol-induced MMP-9 expression of LOVO cells. We also demonstrated that the propofol-induced decrease in invasive ability via ERK1/2 down-regulation was mediated mainly through the GABA-A receptor. These results indicate that propofol stimulation inhibits cancer cell invasion and that the effect is partly due to ERK1/2-dependent down-regulation of MMPs.

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1. Introduction

Colorectal cancer (CRC) is a common form of cancer and a major cause of cancer death worldwide. The incidence of CRC has been rapidly increasing in recent years. Although the incidence of CRC was substantially lower in Asia than in the USA in the mid-twentieth century, the incidence in Japan and China has been rapidly increasing [1,2]. Chemotherapy, immunotherapy, radiation and surgery are common therapies for cancer clinical treatment. Anaesthesia has an important role in almost all of the above procedures, particularly during surgery. At present, many anaesthetic agents are used, during cancer resection surgery, for example, without knowing their effects on cancer, mutagenic potential, impaired metastatic capability, and growth of preexisting tumour cells [3,4].

Propofol (2,6-diisopropylphenol), one of the most commonly used intravenous anaesthetic agents, producing smooth induction and rapid recovery from anaesthesia, and with a limited number of side effects, is one of the anaesthetic agents known to interact with γ-aminobutyric acid A receptor (GABAAR) [5]. It has been shown that propofol can positively modulate and directly activate the

GABAAR channel complex [6]. However, the intracellular response to the interaction between GABAAR and propofol is still not clear [7].

Several studies have been reported that propofol can depress the immune response by significantly inhibit chemotaxis, phagocytosis, and reactive oxygen species production of neutrophils in a dose-dependent manner [8,9]. Moreover, clinically relevant concentrations of propofol are shown to increase migration of MDA-MB-468 breast carcinoma cells by activation of GABAAR [10]. However, opposite results suggested that clinically relevant concentrations of propofol can inhibit the invasion of human cancer cells by modulating Rho A [11]. In particular, it has been observed that in mice, a propofol infusion of 40 mg/kg per day, for 4 weeks, prevents pulmonary metastasis of cancer cells by inhibiting the invasion activity of cancer cells rather than by inhibiting their growth. Moreover, Kushida et al. found that this anaesthetic agent has a beneficial effect on antitumour immunity via greatly enhancement of cytotoxic T lymphocytes activity in vitro in mice [12]. In addition, unlike other anaesthetics, propofol does not reduce natural killer activity or increase MADB106 lung tumour retention or lung metastases [13].

In spite of all of this, to date, few papers have been published specifically addressing a possible correlation between propofol and cancers, and the results are often confused and disputed. Thus, it is surely important to clarify the effects of propofol on the behavior of

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cancer cells. The aim of this study was to investigate whether exposure to propofol influences the invasive ability of human colon carcinoma cells.

2. Materials and methods

2.1. Cell lines

LOVO colon cancer cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI 1640 media (Sigma, St. Louis, USA), supplemented with 10% fetal bovine serum and 100 units/mL of penicillin and streptomycin at 37 °C in a humidified 5% CO_2.

2.2. Reagents and chemicals

Propofol was purchased from Aldrich (Milwaukee, WI). Propofol was diluted in dimethyl sulfoxide (DMSO) for in vitro assays. SB203580, JNK inhibitor II, and PD98059 were from Calbiochem (San Diego, Califonia, USA). All pharmacological inhibitors were determined in preliminary dose-response experiments. Each inhibitor was introduced 1 h before application of a pulse of propofol. For Western blot analysis, the following antibodies were used: rabbit monoclonal antibody against phospho-p38, mouse monoclonal antibody against p38, rabbit monoclonal antibody against phospho-44/42MAPK, and mouse monoclonal antibody against 44/42MAPK (all from Cell Signaling Technology Inc., Beverly, Massachusetts, USA). For detection, an enhanced chemiluminescence reagent detection system (NEN, Boston, Massachusetts, USA) was used.

2.2.1. Reverse transcription, polymerase chain reaction (RT–PCR)

Total RNA was extracted using Trizol reagent (Invitrogen). Reverse transcription reaction was performed using 2 μg of total RNA with a first strand cDNA kit (Takara, Shiga, Japan), according to the manufacturer's instructions. Polymerase chain reaction was run in a 25 μL volume containing 2 μL of cDNA template, 10 × Buffer, 0.15 mM dNTP, 0.1 mM each primer and 0.5 U of Ex Taq Hot Start Version (Takara). The primer sequences are referred to in the reference [14]. The final products were identified in 2% agarose gel and stained with ethidium bromide.

2.3. Transwell assay

Invasion assays were done using a modified Transwell chamber system as described previously [15]. Cells (2×10^5) were seeded on Matrigel-coated (500 ng/mL) membrane inserts with a pore size of 8 μm (BD Bioscience, Heidelberg, Germany) in the presence of RPMI 1640 supplemented with 10% FBS. The same medium of 750 μL was placed in the lower wells of the chamber system. Thereafter, the cells were incubated for 24 h in the absence or presence of propofol(2, 5, 8 μg/mL). The cells on the upper side were scraped off with a rubber policeman and the cells that had migrated into the lower compartment were fixed (4% paraformaldehyde in phosphate buffered saline, PBS), stained with hematoxylin and eosin, and counted from five random high power fields at 200 magnification in each well.

2.4. Immunofluorescence assay

LOVO cells (5×10^4 cells/mL) were grown on coverslips in six-well plates, with or without propofol stimulation, at various concentrations. The cells were washed with cold PBS, fixed in 4% paraformaldehyde/cold PBS, and blocked with 3% bovine serum albumin (BSA)/PBS, followed by detection of metalloproteinases

(MMPs)(MMP-2, and -9). After incubation with primary antibodies against MMP-2, and -9 (Santa Cruz, California, USA; 1:100 dilution) overnight at 4 °C, the cells were incubated with a secondary antibody (Alexa Fluor 488 donkey anti-goat IgG, 1:250 dilution; Molecular Probes, Eugene, Oregon, USA). Samples incubated without the primary antibody were used as negative controls. Immunoreactivity of each sample was observed using a fluorescence microscope (Olympus, Tokyo, Japan).

2.5. Detection of the GABA-A receptor pathway by GABA-A receptor modulation using GABA-A receptor agonist and antagonist

The receptor modulation study was carried out as described previously [16]. The LOVO cells were incubated with the GABA-A receptor agonist propofol at 8 μg/mL for 24 h. Invasive ability was assessed by the matrigel invasion assay, as described above. Receptor blocking was assessed by using the GABA-A receptor antagonist BMI (Sigma Aldrich). Cancer cells were pretreated with receptor antagonist for 1 h at a concentration of 100 μM, and then incubated with propofol (8 μg/mL) for 24 h.

2.6. Gelatin zymography

LOVO cells were plated onto 10 cm culture plates and were incubated with or without different concentrations of propofol at 37 °C for the indicated time. When inhibitors were used, they were added 1 h prior to the application of propofol. After treatment, the culture medium was collected and centrifuged at 1500 rpm for 5 min at 4 °C to remove cell debris. The supernatant was mixed with 5 × non-reducing sample buffer (4:1, v/v) and 20 μg were separated on 10% sodium doecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 1% gelatin as the protease substrate. The procedure was followed by incubation in zymogram renaturation buffer [2.5% Triton X-100] for 1 h at room temperature to remove SDS. The buffer was then replaced with zymogram development buffer [50 mM Tris, 40 mM HCl, 200 mM NaCl, 5 mM CaCl2, and 0.2% Briji 35], and incubation continued overnight on a rotary shaker. After incubation, gels were stained in 30% methanol, 10% acetic acid, and 0.5% (w/v) Coomassie Brilliant Blue for 1 h followed by destaining. Mixed human MMP-2 and MMP-9 were used as positive controls. Gelatinolytic activity was manifested as horizontal white bands on a blue background.

2.7. Western blot analysis

For SDS-PAGE, LOVO cells were plated in 10 cm dishes, and grown at 70–80% subconfluence. The cells were washed with cold PBS and collected by centrifugation, then harvested in RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF,1 mM Na_3VO_4, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. For detection of phosphorylated proteins, the cells were harvested in NP-40 buffer [1% NP-40, 0.5% sodium deoxycholate, 5 mM EDTA, 50 mM Tris HCl (pH 8 0), 0.15 M NaCl], containing 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Protein concentrations were determined with a BCA Protein Assay Kit (PIERCE, Rockford, Illinois, USA). Equal protein amounts (10–40 μg) were run on polyacrylamide gels. For immunoblotting, the proteins were blotted onto polyvinylidene difluoride membranes. The blotted filters were blocked for 1 h at room temperature in PBS with 0.05% Tween-20 containing 5% non-fat milk or 5% BSA, then probed with primary antibodies against phosphorylated p38, p38, phosphorylated ERK1/2, and ERK1/2. After incubation with peroxidaseconjugated anti-rabbit or

anti-mouse IgG, specific reactions were revealed with enhanced chemiluminescence (ECL) reagent (Perkin-Elmer Life Sciences, Boston, Massachusetts, USA).

2.8. Statistical analysis

The results were evaluated by Student's *t* test for paired or nonpaired data, as appropriate. Statistical significance was defined as a value of $P < 0.05$. Values are reported as mean ± SE.

3. Results

3.1. Expression of GABA receptor on colon carcinoma cells, including LOVO, HT29, and SW1116 cells

We evaluated GABA-A receptor expression in the human colon carcinoma cell lines LOVO, HT29, and SW1116 using RT-PCR analysis. As seen in Fig. 1A, three colon carcinoma cell lines expressed GABA-Aβ3 receptor. The results indicated that all cell lines expressed the GABA-A receptor.

### 3.2. MMP down-regulation by propofol stimulation

We examined the influence of propofol stimulation on MMP expression in CRC cells. We performed immunocytochemical staining of MMPs (MMP-2 and -9) on cancer cells after 24 h of propofol stimulation. For this purpose, colon carcinoma cell line LOVO, which possess the most common histological feature in human CRC, were selected. The staining revealed that expression of

MMP-2 and MMP-9 was significantly decreased by propofol stimulation in LOVO cells (Fig. 1B). To further clarify this phenomenon, gelatin zymography was introduced (Fig. 1C). The LOVO cells were incubated with GABA-A receptor antagonists as described in Materials and Methods. Cells without any treatment served as negative controls, and mixed human MMP-2 and MMP-9 were used as positive controls. Gelatinolytic activity was manifested as horizontal white bands on a blue background (Fig. 1C).

Expression of MMP-2 and -9 was significantly decreased by propofol stimulation in comparison with the negative controls (MMP2 expression in cells treated with propofol, mean ± SE = 2.63 ± 0.49 times lower vs. control, $P < 0.05$; MMP9, mean ± SE = 4.46 ± 0.57 vs. control, $P < 0.01$: Fig. 1C).

3.3. Sustained inactivation of ERK1/2 by propofol treatment potently down-regulates MMP-9

It has been reported previously that expression of MMPs is regulated by various kinds of protein kinase [17–19]. In order to elucidate the particular signaling pathways involved in GABA-A receptor mediated down-regulation of MMP-2 and -9, we further evaluated the activity of ERK1/2, p38 MAPK, and c-Jun Nterminal kinase (JNK) in propofol-treated LOVO cells (Fig. 2A). ERK1/2 phosphorylation, an activated form of ERK1/2, was significantly decreased in LOVO cells as early as 10 min after treatment with propofol, and this effect reached a maximum at 30 min, persisting for 12 h (panel a in Fig. 2A). On the other hand, deactivation of p38 MAPK or JNK, which also occurred within 10 min after propofol
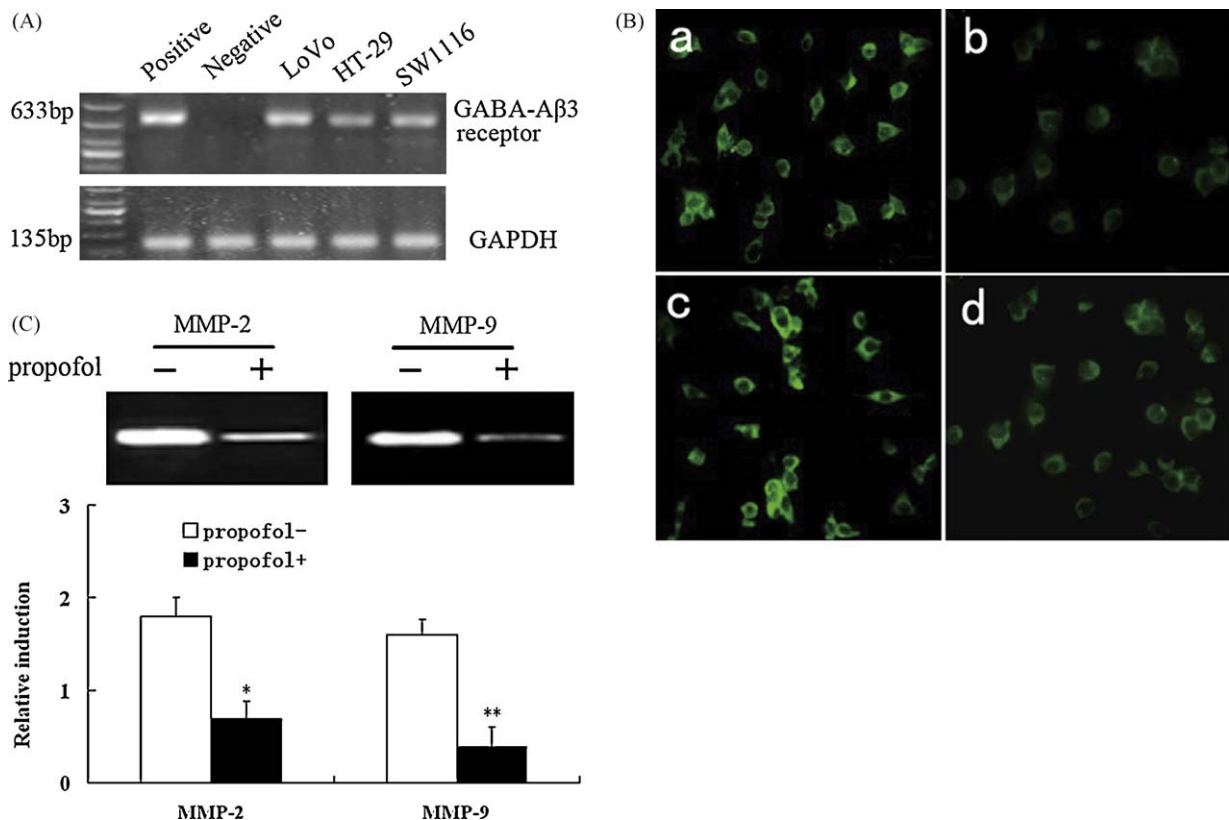
Fig. 1. Propofol treatment induces down-regulation of MMPs. A. RT-PCR analysis for GABA-Aβ3 receptor using total RNA from LOVO, HT29, and SW1116 cells. GAPDH was used as an endogenous control. GABA-Aβ3 receptor subunit is expressed by all these cell lines. B. Expression of MMP-2 or -9 in LOVO cells with or without propofol treatment (8 μg/mL) for 24 h. Expression of MMP-2 in control cells (a), or cells treated with propofol (b) and expression of MMP-9 in control cells (c) or cells treated with propofol were examined by immunofluorescence. Bar indicates 50 μm. C. Expression of MMP-2 or -9 in LOVO cells (1 × 10⁶ cells) incubated with propofol (8 μg/mL) for 24 hexamined by Gelatin zymography. The upper panel shows the representative pictures of MMP-2 or -9 in cell treated with or without GABA. The lower panel shows quantitative evaluation of MMP-2 or -9 treated with or without GABA. Each value was determined by NIH-image, and normalized to the expression of MMP-2 or -9 without propofol incubation, and represents a mean of triplicate determinants; bars correspond to standard deviation. Asterisks (*, **) indicate statistically significant; ns indicates nothing significant.
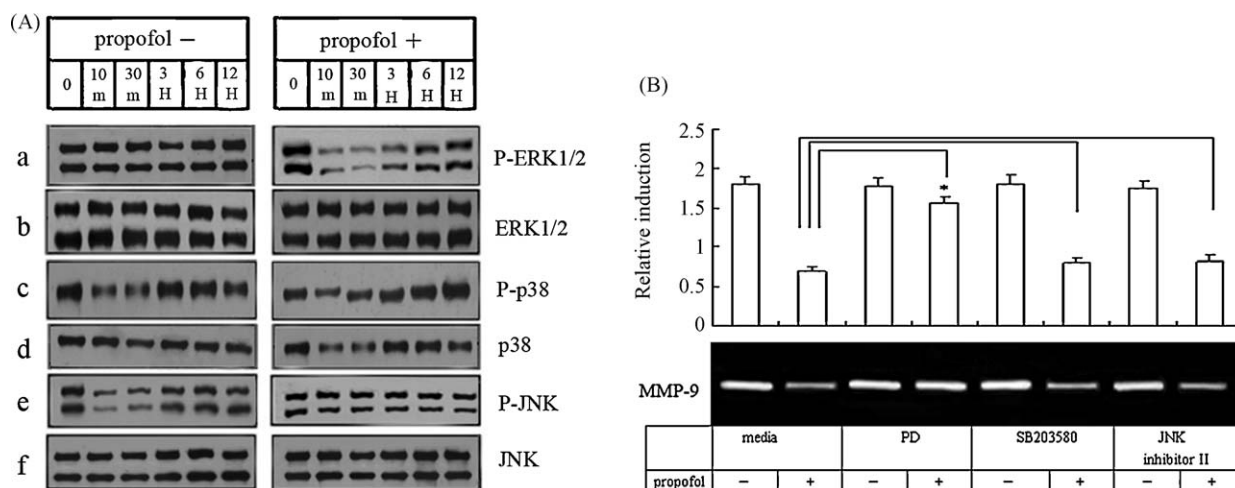
Fig. 2. Propofol induced MAPK deactivation. A. Expression of phospholyrated/total formof ERK1/2, JNK, or p38 in LOVO cells with or without propofol treatment (8 μg/mL), at indicated time. B. LOVO cells were pretreated with PD98059, SB203580, or JNK specific inhibitor, then incubated with media only (media), or propofol. MMP-9 expression was evaluated by gelatin zymography. Semi-quantitative analysis of the blots was performed by densitometry. Results were expressed as relative values to control, and represent a mean of triplicate determinants; bars correspond to standard deviation. Asterisk (*) indicates statistically significant; ns indicates nothing significant.

treatment, returned to the endogenous level at 30 min (panel c in Fig. 2A). Total protein levels of ERK1/2, p38, and JNK were not altered by propofol treatment (panels b, d, f in Fig. 2A). To further characterize the specific pathway that plays a key role in the propofol induced depression of MMP-9 expression, specific inhibitors against each pathway were employed (Fig. 2B). LOVO cells treated with a selective inhibitor of the ras/raf/MEK/ERK pathway, PD98059, exhibited marked abrogation of propofol - induced MMP-9 down-regulation (propofol + PD98059, mean ± SE = 1.00 ± 0.05 times higher vs. control, propofol only, mean ± SE = 0.36 ± 0.03 times higher vs. control, $P < 0.01$: Fig. 2B). In contrast, treatment with the p38 inhibitor, SB203580, had a much less marked effect on propofol-induced MMP-9 expression (propofol + SB203580 vs. propofol only = mean ± SE = 0.41 ± 0.03 times higher vs. 0.36 ± 0.03 times higher, $P = 0.40$). Treatment with the JNK-specific inhibitor, JNKi, had almost no effect on propofol-induced MMP-9 expression (propofol + JNKi vs. propofol only = mean ± SE = 0.39 ± 0.03 times higher vs. 0.36 ± 0.02 times higher, $P = 0.49$: Fig. 2B). These results appear to confirm that ERK1/2 is relevant to the

down-regulation of MMP-9 due to propofol treatment, rather than p38MAPK and JNK.

3.4. Decreased invasive ability stimulated by propofol and possible involvement of GABAAR pathway

Recent observations suggest that MMPs play an important role in promoting cancer cell invasion [20,21]. We examined the influence of propofol stimulation on the invasive ability of cancer cells by in vitro invasion assay using a BioCoat Matrigel Invasion Chamber Kit. A filter coated with Matrigel was used as a model of the basement membrane. LOVO cells were incubated with propofol at various concentrations (0, 2 μg/mL, 5 μg/mL, and 8 μg/mL), and cells that invaded to the lower compartment of the chamber through the filter were counted after 24 h of incubation. The number of invaded cells was decreased significantly by propofol stimulation in a dose-dependent manner. However, the treatment with GABAAR antagonist bicuculine sufficiently abrogated the inhibition of cancer invasion by propofol (number of invaded cells = 79.3 ± 4.61 in controls, 68.17 ± 3.59 with 2 μg/mL propofol treatment, 35.33 ± 2.69 at 5 μg/mL propofol*, 24.66 ± 2.11 at 8 μg/ mL propofol**, and 70.5 ± 3.86 at 8 μg/mL propofol with pretreatment of 100 μM bicuculine: Fig. 3).

4. Discussion

In the present study, we have demonstrated that propofol decreases expression of MMP-2 and -9 of human colon carcinoma Cell line LOVO, through the suppression of ERK1/2 pathway activation, thereby inhibiting cancer cell invasion.

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (CNS), and is synthesized from L-glutamate by L-glutamate decarboxylase in the CNS and by diamine oxidase in the periphery [22,23]. GABA and its receptors have been found in nonneuronal peripheral tissues such as the gastrointestinal system, lung, liver, and testes [24]. GABA acts on either ionotropic (GABAA or GABAC) or metabotropic (GABAB) receptors [25,26].

GABAAR is a major mediator of rapid synaptic inhibition in signal transmission, as well as an important drug target. It consists of a pentameric complex, formed by different subunits (α1–6, β1– 4, γ1–4, δ, ε, θ), and contains an integral chloride channel. Binding
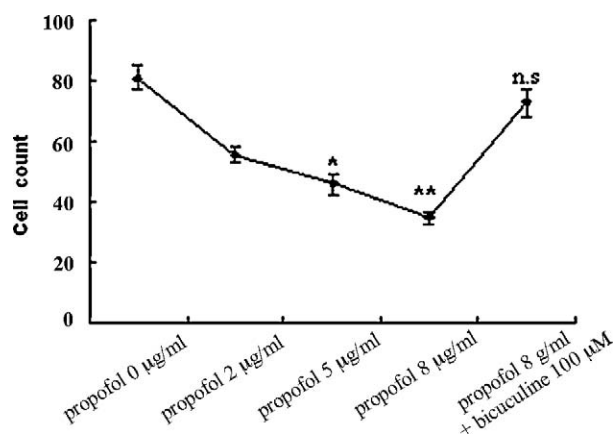
Fig. 3. Propofol induced impairment on invasive ability of LOVO cells. Dose-dependent manner of inhibition of LOVO on invasive ability of LOVO cells by propofol. This effect was abrogated by pretreated with GABAAR antagonist bicuculine (100 μM). LOVO cells were placed into the upper chamber with propofol at the indicated concentrations. The number of cells that had invaded through the filter were counted after 24 h of incubation. The results were expressed as a mean of triplicate determinants; bars correspond to standard deviation. Asterisks (*, **) indicate statistically significant; ns indicates nothing significant.

of a GABAAR agonist or anaesthetic agent results in an influx of chloride ions, leading to hyperpolarization of the cell and an unresponsiveness of the cell to external stimuli [27]. A high concentration of neurotransmitter-gated ion channels is a prerequisite for rapid pre-synaptic transmission. GABA receptors containing β3 subunits are important mediators of propofol anaesthetic effects in vivo, because it has been shown that mice with the knock-in point mutation β3N265 M lack the immobilizing effects of propofol in response to painful stimuli [28]. In the present study, all colon carcinoma cell lines used in the experiment expressed the GABA-Aβ3 receptor, showing a remarkable finding of this study–the close relationship between GABAA receptor and the biology of colon carcinoma cells.

The development of cancer invasion and metastasis is a complex cascade of events involving tumor dissemination from the primary site to distant organs. The cancer cells must separate from the primary tumor, invade the stromal tissue, enter the circulation, extravasate, and invade the target organ, forming a metastatic colony. During this process, the degradation of extracellular matrix (ECM), which exerts biochemical and mechanical barriers to cell movement has been shown to be an important biological process in the metastasis of cancer cells. Excess ECM degradation is one of the hallmarks of tumor metastasis, and matrix MMPs are shown to be crucial proteinases that enable tumor cells to permeate the basement membrane and invade the surrounding tissues [29–32]. MMPs were revealed not only cleave matrix components but may also have functions to release bioactive peptides such as growth factors and growth factor-binding proteins at a site of extracellular matrix remodeling [33]. MMP-2 and MMP-9 (also known as type IV collagenases or gelatinases) can degrade most ECM components forming the basal membrane [34]. In the present study, exposure to propofol of clinically relevant concentrations significantly decreased the production and activity of MMPs. In addition, the invasive ability of LOVO cells was impaired by propofol, and this effect was abrogated by GABAAR antagonist bicuculine sufficiently. These results may be strong evidence that propofol inhibit the invasion of cancer cells through GABAAR pathway.

To further clarify the mechanism involved in the inhibition of invasive ability of LOVO cells, we investigated the effect of propofol on intracellular pathway of Mitogen-activated protein kinases (MAPKs). MAPKs are known to be important mediators of signal transduction from the cell surface to the nucleus [35]. There are three major mammalian MAP kinases, including extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK. The diverse MAP kinase members are activated in response to different extracellular stimuli and have distinct downstream targets, thus serving different roles in cellular responses. ERK1/2, p38 MAPK, and JNK/SAPK play a central role in regulating the expressions of MMPs [36–38]. How anaesthetic agent propofol affects this pathway is still poorly defined. Our in vitro study showed that all major MAPKs tested (ERK1/2, p38, and JNK) were deactivated by propofol treatment, and that deactivation of ERK1/2 were sustained by propofol stimulation and suppressed MMP production.

Based on these results, the major finding of our present study is that GABAAR agonistic anaesthetic agent propofol inhibits cancer cells invasive ability by impairment of the MMP production. Joseph et al. established that the inhibitory effect of GABA on colon cancer cells is mediated via the GABAB receptor pathway by the regulation of cellular cAMP that in turn decreases the colon cancer cells' ability to migrate [39]. In contrast, recently Inamoto et al. reported that in renal cancer the GABAB receptor pathway induced cancer cell invasion and metastasis by promoting matrix metalloproteinase production [40]. Different cell types and/or

different extracellular conditions may explain the discrepancy between these results. It is possible that GABA-associated receptors may have different effects depending on the origin of the tumor. More importantly, these studies highlight the complexity of the GABA receptor pathway. Although further investigation is necessary to clarify the exact mechanisms or functioning action of propofol on cancer metastasis, we propose that propofol influences the invasive ability of cancer cells.

In conclusion, we found that clinically relevant concentrations of propofol stimulation may inhibit cancer cell invasion, and that a decreased MMP production caused by GABA-A receptor pathway mediated MAPKs deactivation, especially ERK1/2, may profoundly be involved in the process. These findings show that propofol might be an ideal anaesthetic for cancer surgery and the GABAAR-ERKs-MMP cascade is expected to be a possible target to be utilized in the future treatment of CRC.

Conflict of interest statement

The authors have declared that no conflict of interest exists.

Acknowledgments

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