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Full Length Research Paper

Effects of midazolam, diazepam, propofol and dexmedetomidine on endothelial cell proliferation and angiogenesis induced by VEGF

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The goal of this study was to investigate the effects of the sedative drugs midazolam, diazepam, propofol and dexmedetomidine on cell proliferation and vascular endothelial growth factor (VEGF)-induced angiogenesis. The effects of the drugs on cell proliferation and angiogenesis were evaluated using human umbilical vein endothelial cells (HUVECs). HUVECs were exposed to 200 ng/ml of midazolam, 600 ng/ml of diazepam, 3 µg/ml of propofol, or 1 ng/ml of dexmedetomidine (clinically relevant concentrations) for 1, 3, 5 or 10 days in the presence of 10 ng/ml of VEGF. Values are expressed as means ± SE, with statistical analysis performed by ANOVA and Bonferroni-type multiple *t*-tests. Treatment of HUVECs with 200 ng/ml of midazolam for 10 days and 600 ng/ml of diazepam for 5 or 10 days resulted in significant inhibition of cell proliferation and angiogenesis ($P < 0.05$, vs. controls), whereas propofol and dexmedetomidine did not inhibit proliferation or angiogenesis over any time period. These results suggest that propofol and dexmedetomidine can be used safely for long term sedation in patients receiving therapeutic angiogenesis for ischemic vascular disease, whereas midazolam and diazepam should not be used, since they may inhibit angiogenesis in these patients.

Key words: Proliferation, angiogenesis, midazolam, diazepam, propofol, dexmedetomidine.

INTRODUCTION

Angiogenesis involves several steps, including endothelial cell proliferation, cell migration and tube formation (Bratt et al., 2005). Vascular endothelial growth factor (VEGF) is a key regulator of most factors responsible for inducing angiogenesis, including those that regulate endothelial cell proliferation, permeability and survival. The effectiveness of angiogenesis therapy has been shown by intramyocardial injection of VEGF in rats (Sung et al., 2009) and intracoronary injection of plasmid VEGF165 in dogs (Furlani et al., 2009). Furthermore, VEGF 165 promotes cardiomyogenesis in chronic myocardial ischemia and nonreperfused myocardial infarction (Guerrero et al., 2008). Hence, induction of angiogenesis is an attractive new therapeutic strategy for patients suffering from critical ischemic disease.

Midazolam and diazepam are benzodiazepines that are commonly used in clinical anesthesia and in the intensive care unit (ICU) (Young and Prielipp, 2001; Payen et al., 2007); these drugs induce sedation via potentiation of the inhibitory neurotransmitter gamma amino-butyric acid (GABA) (Stevanovic, 2006). Propofol has a short-acting sedative/hypnotic effect through the GABA receptor (Payen et al., 2007) and dexmedetomidine has a highly specific and selective α_2-adrenergic receptor agonistic effect and is widely used as an anesthetic or sedative adjuvant (Lucchinetti et al., 2007; Shukry and Ramadhyani, 2005; Scheinin et al., 1998; Tobias, 2007).

Sedation is an important part of therapy for critically ill patients in the ICU. Although midazolam, diazepam, propofol and dexmedetomidine are widely used for sedation, the effects of these drugs on cell proliferation

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and angiogenesis at clinically relevant concentrations (plasma concentrations observed clinically) have not been studied. Therefore, we investigated the effects of the clinically relevant concentrations of midazolam, diazepam, propofol, dexmedetomidine on human endothelial cell proliferation and angiogenesis induced by VEGF, using an *in vitro* culture system.

MATERIALS AND METHODS

Materials

A cell counting kit-F was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan) and an angiogenesis kit was obtained from Kurabo Co. (Osaka, Japan). Midazolam and diazepam were purchased from Astellas Pharmaceutical Inc. (Tokyo, Japan) and propofol and dexmedetomidine were purchased from Maruishi Pharmaceutical Co. Ltd. (Osaka, Japan). Cell culture plates (96- and 24-well) were obtained from Becton Dickinson Biosciences (Franklin Lakes, NJ, USA), Trypsin/EDTA was obtained from GIBCO Life Technologies (Eggenstein, Germany) and EGM-2 culture medium was purchased from Cambrex Bio Science (Walkersville, MD, USA). PBS solution was composed of 137 mmol NaCl, 2.7 mmol KCl, 0.2 mmol $Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4, all from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Walkersville, MD, USA) and cultured in EGM-2 culture medium in a humidified atmosphere (5% CO_2 + 95% air) at 37°C for the cell proliferation assay.

Cell proliferation assays

HUVECs were trypsinized and plated in 96-well plates at 2500 cells per well in 100 µL EGM-2 culture medium and incubated at 37°C in 5% CO_2 + 95% air. After the EGM-2 culture medium was changed on the following day, the cells were exposed to 200 ng/ml of midazolam, 600 ng/ml of diazepam, 3 µg/ml of propofol, or 1 ng/ml of dexmedetomidine (clinically relevant concentrations) in each well, and incubated for 1, 3, 5 or 10 days to examine cell proliferation. All cells were incubated for 11 days with or without sedative drugs, and on the 11th day the cell number was quantified using cell counting kit-F. To determine the number of viable cells per well, the fluorescence per well was determined using a spectrophotometer (Fluoro Light FP-3000, Nition Corp., Chiba, Japan) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

In vitro angiogenesis assay

Evaluation of angiogenesis using an *in vitro* angiogenesis kit was performed by assessing the number of micro vessel structures formed by HUVECs pre-seeded in a 24-well plate and co-cultured with fibroblasts cells at 37°C in 5% CO_2 + 95% air for 11 days with or without sedative drugs. Separate wells were exposed to 200 ng/ml of midazolam, 600 ng/ml of diazepam, 3 µg/ml of propofol, or 1 ng/ml of dexmedetomidine for 1, 3, 5 or 10 days. Control cells were not treated with sedative agents and negative-control cells were treated with suramin, an angiosuppressive agent. All wells contained 10 ng/ml of VEGF.

Neo-vessel staining and counting

On day 11 of incubation, the 24-well cell culture plates were washed with PBS, fixed with 1 ml of 70% cold ethanol and then washed with PBS containing 1% bovine serum albumin (BSA). The wells were incubated with the primary antibody, a mouse anti-human antibody against CD31 (supplied with the kit, diluted 1:4000), for 1 h at 37°C and washed with PBS. Then the wells were incubated with the secondary antibody, an alkaline phosphatase-conjugated goat anti-mouse IgG (diluted 1:500) for 1 h at 37°C and washed with distilled water. For vascular wall staining, 0.5 ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) solution were added to the wells and incubated at 37°C until appearance of a purple color (5 - 10 min). Neovascularization was assessed using a Chalkley Grid Lens for scoring neo-vessels (tubules) under an Olympus CKX41 microscope (Olympus, Tokyo, Japan) at 40-fold magnification.

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) with Bonferroni-type multiple *t*-tests, with levels of 0.05 or 0.01 considered to indicate significant differences. Values are expressed as means ± SE.

RESULTS

Proliferation

Midazolam and diazepam significantly inhibited proliferation of HUVECs, but propofol and dexmedetomidine did not inhibit proliferation (Figures 1A, B, C and 1D). With 1- and 3-day exposure times, none of the drugs significantly inhibited proliferation of HUVECs. With 5-day exposure to midazolam, diazepam, propofol, dexmedetomidine and no drug (control), the cell counts were 22576 ± 1657, 20699 ± 1478, 27734 ± 2326, 27699 ± 2988 and 29513 ± 2464, respectively, indicating that only diazepam significantly inhibited proliferation of HUVECs ($P < 0.05$ vs. control). With 10-day exposure, the cells counts were 19936 ± 1788, 18888 ± 2047, 26929 ± 2326, 27185 ± 2287, and 29513 ± 2464, respectively, with midazolam and diazepam significantly inhibiting HUVEC proliferation ($P < 0.05$ and $P < 0.01$ vs. control). Propofol and dexmedetomidine showed no inhibition of HUVEC proliferation over any time period.

Effect of midazolam on VEGF-induced angiogenesis

When HUVECs were exposed to 200 ng/ml of midazolam for 1, 3, 5 and 10 days, the tubule scores were 51.1 ± 1.1, 47.3 ± 1.1, 46.5 ± 3.2, and 43.3 ± 3.2, respectively and the tubule scores of non-treated controls and suramin-treated negative controls were 53.1 ± 1.8 and 30.5 ± 1.8, respectively. Therefore, exposure to 200 ng ml^{-1} of midazolam for 10 days caused significant inhibition of angiogenesis induced by 10 ng/ml of VEGF ($P < 0.05$ vs. control; Figure 2(A and B).

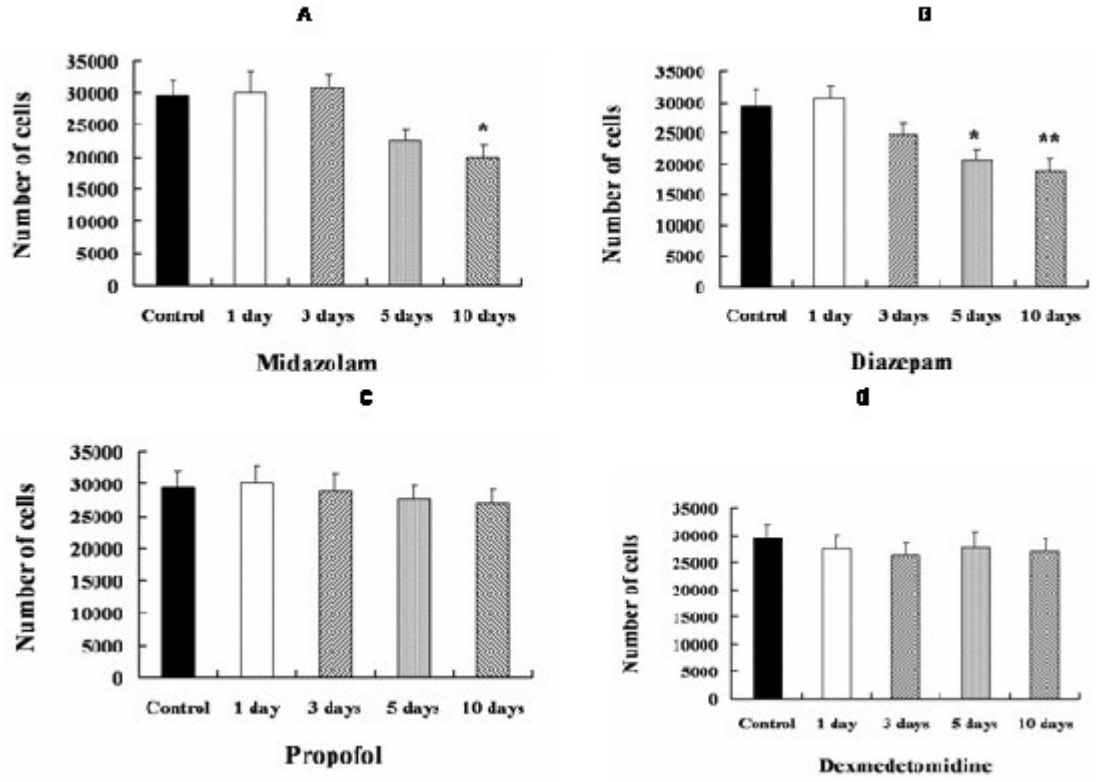

Figure 1. Effects of midazolam, diazepam, propofol, and dexmedetomidine on the proliferation of human umbilical vein endothelial cells (HUVECs). Cells were treated for 1, 3, 5 or 10 days with 200 ng/mL of midazolam (1A), 600 ng/mL of diazepam (1B), 3 µg/mL of propofol (1C), or 1 ng/mL of dexmedetomidine (1D). Cell numbers were quantified using cell counting kit-F. Data are shown as means ± SE values; n = 8. * and **: P < 0.05 and 0.01, respectively, vs. control cells (no drug treatment).

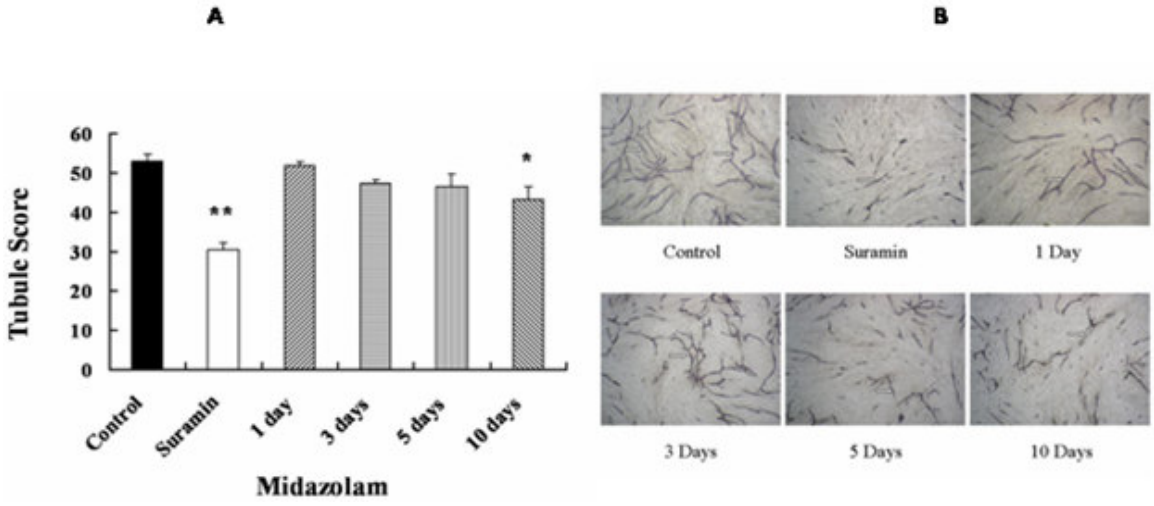

Figure 2. Quantification of tubule score (2A) and neovascularization (2B) for cells treated with 200 ng/mL of midazolam for 1, 3, 5 or 10 days. Tubule scores were counted with a Chalkley Grid Lens and the center of the field in each well was photographed (*: P < 0.05; **: P < 0.01). Data are shown as means ± SE values; n = 8. The arrows indicate angiogenesis.

Effect of diazepam on VEGF-induced angiogenesis

When HUVECs were exposed to 600 ng/ml of diazepam for 1, 3, 5 and 10 days, the tubule scores were 51.8 ± 1.5, 46.3 ± 1, 43.5 ± 3, and 41.5 ± 2.6, respectively, and the tubule scores of non-treated controls and suramin-

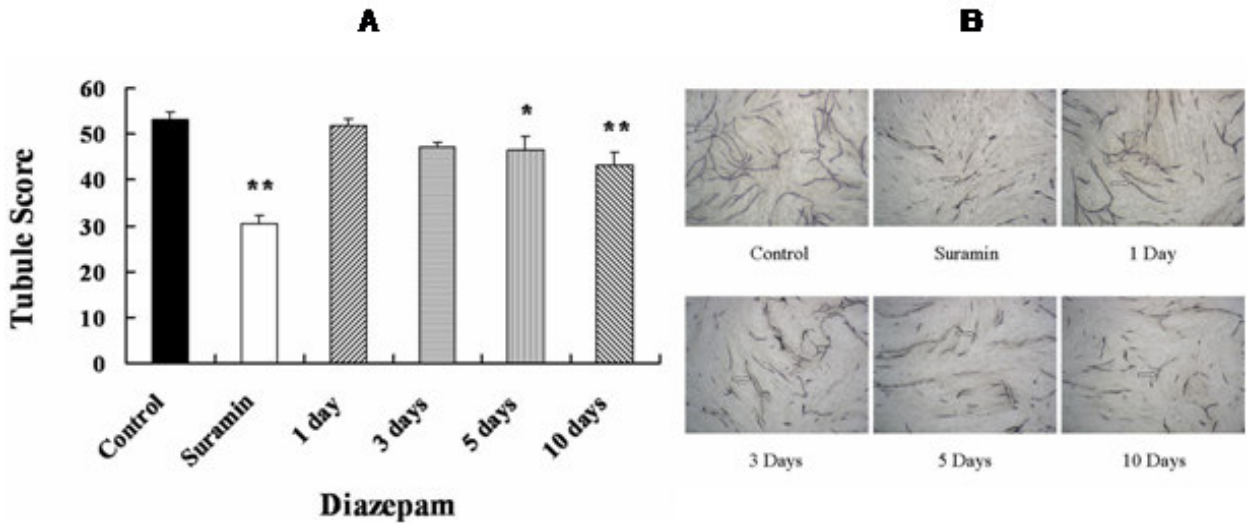

Figure 3. Quantification of tubule score (3A) and neovascularization (3B) for cells treated with 600 ng/mL of diazepam for 1, 3, 5 or 10 days. Tubule scores were counted with a Chalkley Grid Lens and the center of the field in each well was photographed (*: P < 0.05; **: P < 0.01). Data are shown as means ± SE values; n = 8. The arrows indicate angiogenesis.

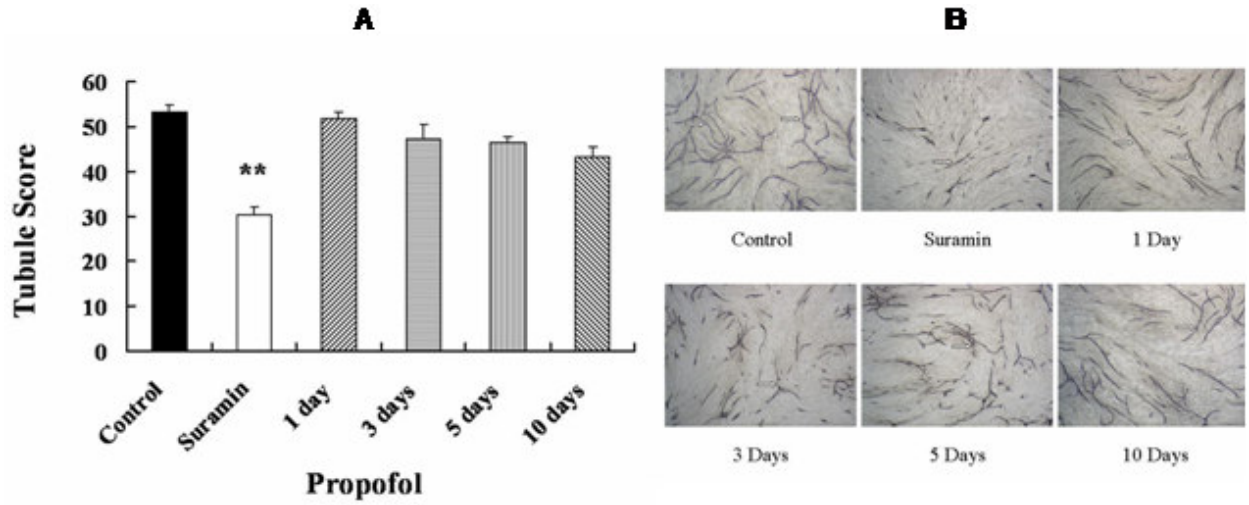

Figure 4. Quantification of tubule score (4A) and neovascularization (4B) for cells treated with 3 µg/mL of propofol for 1, 3, 5 or 10 days. Tubule scores were counted with a Chalkley Grid Lens and the center of the field in each well was photographed (**: *P* < 0.01). Data are shown as means ± SE values; *n* = 8. The arrows indicate angiogenesis.

treated negative controls were 53.1 ± 1.8 and 30.5 ± 1.8, respectively. Therefore, exposure to 600 ng/ml of diazepam for 5 or 10 days caused significant inhibition of angiogenesis induced by ng ml^{-1} of VEGF (P < 0.05 and 0.01 vs. control; Figures 3 A and B).

Effect of propofol on VEGF-induced angiogenesis

When HUVECs were exposed to 3 µg/ml of propofol for 1, 3, 5 and 10 days, the tubule score were 51.8 ± 1.6, 51.3 ± 3.1, 51.8 ± 1.3 and 50.5 ± 2.1, respectively, and the tubule scores of non-treated controls and suramin-treated negative controls were 53.1 ± 1.8 and 30.5 ± 1.8, respectively. Therefore, propofol did not significantly inhibit angiogenesis induced by 10 ng/ml of VEGF (Figure 4A and B).

Effect of dexmedetomidine on VEGF-induced angiogenesis

When HUVECs were exposed to 1 ng/ml of dexmedetomidine for 1, 3, 5 and 10 days, the tubule

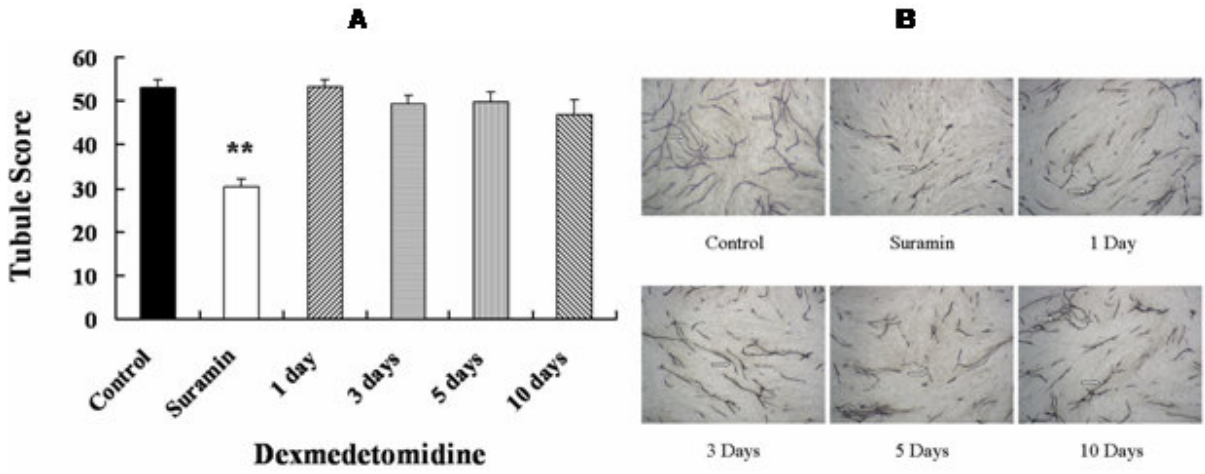

Figure 5. Quantification of tubule score (5A) and neovascularization (5B) for cells treated with 1 ng/mL of dexmedetomidine for 1, 3, 5 or 10 days. Tubule scores were counted with a Chalkley Grid Lens and the center of the field in each well was photographed (**: $P < 0.01$). Data are shown as means ± SE values; n = 8. The arrows indicate angiogenesis.

scores were 53.3 ± 1.7, 49.3 ± 2.2, 49.8 ± 2.3 and 47 ± 3.2, respectively and the tubule scores of non-treated controls and suramin-treated negative controls were 53.1 ± 1.8 and 30.5 ± 1.8, respectively. Therefore, dexmedetomidine did not significantly inhibit angiogenesis induced by 10 ng/ml of VEGF (Figure 5 (A and B)).

DISCUSSION

In therapeutic angiogenesis, an angiogenic growth factor protein or gene is administered to augment the development of endogenous collateral vessels in the ischemic organ. Therapeutic angiogenesis is a new strategy against critical ischemic disease and the number of patients requiring such therapy has recently increased (Lee and Smits, 2002).

In the present study, the results of the cell proliferation assay were consistent with those of the angiogenesis assay. This reveals a significant association between proliferation of endothelial cells and angiogenesis, suggesting that such cell proliferation constitutes one key event in the angiogenesis process. Vascular endothelial growth factor (VEGF) has a strong angiogenic effect in induction of neovascularization and therapeutic angiogenesis using these properties can be used for treating ischemic disease. VEGF is a potent angiogenic factor that is responsible for an increase in vessel density that leads to faster growth (Claffey and Robinson, 1996). VEGF stimulates capillary formation *in vivo*, has direct and selective mitogenic actions on vascular endothelial cells and may directly stimulate the development of new blood vessels (Morbidelli et al., 2010; Eming and Krieg, 2006).

Exposure to midazolam for 10 days resulted in significant inhibition of cell proliferation and angiogenesis (Figures 1A and 2 (A and B). Midazolam has previously been shown to have no inhibitory effect on proliferation of cultured rat type II pneumocytes (type II alveolar epithetlial cells) (Nishina et al., 2002), but midazolam does have a tendency to inhibit lectin-induced cell proliferation in cultured rat peripheral blood mononuclear cells (Chanimov et al., 2000). Therefore, previous results and those in the current study suggest that the effects of midazolam on cell proliferation may be dependent on the cell type. Bidri et al. (1999) demonstrated that exposure of mucosal-like and serosal-like mouse mast cells for 24 and 48 h to midazolam at higher concentrations (10^{-5} to 10^{-4} mol) resulted in significant and dose-dependent inhibition of proliferation, whereas no inhibition occurred at lower concentrations (10^{-7} or 10^{-6} mol) (Bidri et al., 1999). The inhibitory effect on mouse mast cell proliferation was proposed to occur via proinflammatory mediators upon triggering of high-affinity IgE receptors (Bidri et al., 1999).

Many reports have described an inhibitory effect of diazepam on proliferation of various cell types, similarly to our results (Figure 1B). Diazepam diminishes proliferation of primary astrocyte lines, the human breast cancer cell line BT-20, C6 glioma cells, human melanoma M6 (Nordenberg et al., 1999), rat pituitary tumor cells (Kunert-Radek et al., 1994) and endothelial HECa10 cells (Misztal-Dethloff et al., 2005). However, the concentration of diazepam required to inhibit endothelial cell proliferation was 10^{-4} mol, which is about 50 times higher than the clinically relevant concentration of 600 ng/ml. The antiproliferative activity of diazepam seems to be due to a calcium-dependent process associated with a direct peripheral benzodiazepine receptor (Kunert-Radek et al., 1994).

In the current study, propofol and dexmedetomidine showed no inhibitory action on proliferation and angiogenesis and propofol has previously been shown to

have no inhibitory effect on rat mesangial cell proliferation at doses from 1 to 100 µmol (Jimi et al., 1997), or on rat type II pneumocytes (Nishina et al., 2002). However, a tendency for propofol inhibition of lectin-induced proliferation of cultured rat peripheral blood mononuclear cells has been reported (Chanimov et al., 2000). Dexmedetomidine is a highly specific α_2-adrenergic receptor agonist and it has been shown that α_2-adrenergic receptor activation by dexmedetomidine does not promote cell proliferation, but significantly increases smooth muscle cell migration (Richman et al., 1998). α_2-adrenergic receptors activate MAPK and modulate proliferation of primary cultured proximal tubule cells, which may play a positive role in tubular regeneration (Cussac et al., 2002). However, dexmedetomidine showed neither inhibitory nor stimulatory effects on proliferation and angiogenesis under the conditions of the current study.

There are three VEGF-specific tyrosine kinase receptor subtypes, VEGFR-1, VEGFR-2 and VEGFR-3, with VEGFR-2 thought to be the specific receptor for mediation of angiogenesis (Jain, 2003). However, we were unable to find a study of the effects of midazolam and diazepam on VEGFR-2 and so it is uncertain whether midazolam and diazepam exert an anti-angiogenesis effect through VEGFR-2 and the precise mechanism remains unclear.

To summarize, our results indicate that midazolam and diazepam inhibit cell proliferation and VEGF-induced angiogenesis in human endothelial cells over 11 days of culture, whereas propofol and dexmedetomidine have no such effects. If these results apply in a clinical setting, propofol and dexmedetomidine can be used safely for sedation of patients receiving therapeutic angiogenesis for ischemic vascular disease, but midazolam and diazepam should not be used, since they may inhibit angiogenesis at the clinical dose. However, further clinical studies are needed to clarify the optimal sedative drug for patients undergoing therapeutic angiogenesis for critical limb ischemia and chronic myocardial ischemia.

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