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In vitro evaluation of the angiostatic potential of drugs using an endothelialized tissue engineered connective tissue

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Nonstandard abbreviations used:

ERCT	endothelialized reconstructed connective tissue
HUVEC	human umbilical vein endothelial cells
HMVEC	human microvascular endothelial cells
CLS	capillary-like structures

VEGF vascular endothelial growth factor

MMP matrix metallo proteinase

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Abstract

The development of a new pharmacological strategy, the angiostatic therapy, to inhibit solid tumor progression has increased the need of powerful in vitro models to screen the angiostatic potential of new drug candidates. We produced an endothelialized reconstructed connective tissue (ERCT) that promotes the spontaneous formation of a human capillary-like network by coculture of human endothelial cells isolated from umbilical cord or from newborn foreskin, with dermal fibroblasts in a collagen sponge. Three inhibitors of angiogenesis: Tamoxifen, Ilomastat and Echistatin were used to assess the efficiency of our ERCT to discriminate in vitro an angiostatic potential. The capillary-like structures were characterized by their immunoreactivity to PECAM-1 antibodies and were quantified on histological cross-sections of biopsies taken after 10, 17, 24 and 31 days of culture. A dose-response significant inhibition of the capillary-like formation was detected, when increasing concentrations of Tamoxifen, Ilomastat or Echistatin were added for one week to the culture medium of the ERCT. Tamoxifen was found to be angiogenic at 10 µM and to have a cytotoxic effect at 40 µM, one week after drug removal. Echistatin induced a rapid, slight and reversible inhibition of capillary-like formation, while Ilomastat caused a very precocious, strong and reversible inhibition of angiogenesis. In addition, a 16 hours hypoxia promoted the formation of 10 times larger vessels (> 300 μm^2), compared to normoxic condition. These results suggest that our model could be efficiently used to study the long-term angiostatic potential of drugs *in vitro* in a very physiological environment.

Introduction

Angiogenesis is the production of new capillaries formed by the sprouting or intussusceptive (non-sprouting) growth of endothelial cells from pre-existing blood vessels (Risau, 1997). Angiogenesis leads to activation of endothelial cells, which degrade their basement membrane and surrounding extracellular matrix to migrate through the tissue and proliferate (Wilting and Christ, 1996; Risau, 1997). In adults, angiogenesis is mainly induced after a trauma, such as a wound or an ischemia, or during pathological processes such as retinopathy, leukemia, rheumatoid arthritis, diabetic retinopathy, fibrosis, psoriasis, ulcer and tumor growth (Carmeliet and Jain, 2000; Folkman, 2001). A new therapeutic approach was developed to inhibit the development of solid tumor by blocking their vascularization with an angiostatic drugs (Carmeliet, 2003). The angiostatic strategy is now the most promising way to inhibit angiogenesis dependant diseases, although only few drugs has yet been approved for clinical application (Avastin, an anti-VEGF monoclonal antibody, was approved in 2004 (Kabbinavar et al., 2005)). Several angiogenesis models have been developed in order to investigate the mechanisms of angiogenesis and to allow a better targeting of angiostatic drugs in a pre-clinical step (Cockerill et al., 1995). Various animal models were produced such as the chick chorioallantoic membrane, the rabbit cornea assay, the sponge implant models, the Matrigel plug and conventional tumor models (Auerbach et al., 2003). Nevertheless, these models remain extremely complex, relatively expensive and difficult to interpret for routine screening. Thus, in recent years, a number of *in vitro* models have been developed. The most common *in vitro* models are the three-dimensional gel assays made of various extracellular matrix compounds such as type I collagen, fibrin, fibronectin, basement membrane extracts, or Matrigel™, in which endothelial cells are embedded (Auerbach et al., 2003; Ley et al., 2004; Robinet et al., 2005).

These in vitro models reconstitute an environment made of only one type of extracellular matrix molecule (except for Matrigel), with one type of cells, the endothelial cells, in contrast with the physiological environment, which features several different molecules and cells, that are known to be essential to modulate neovascularization *in vivo* and *in vitro* (Vernon and Sage, 1999). In addition, most of these models are produced with animal endothelial cells (Vernon and Sage, 1999; Auerbach et al., 2003), which have a much lower growth factor requirement to survive, proliferate and form tubular structures (Cavallaro et al., 2001). Moreover, many *in vitro* angiogenesis models achieve the formation of cord-like structures instead of capillary-like ones (Zimrin et al., 1995) and can present a basement membrane on the lumen side (Sage and Vernon, 1994).

We developed a model of endothelialized reconstructed dermis that promotes the spontaneous formation of a capillary-like network by coculture of human umbilical vein endothelial cells (HUVEC) with dermal fibroblasts in a collagen biopolymer (Black et al., 1998; Hudon et al., 2003). In contrast to most *in vitro* angiogenesis models produced with human endothelial cells (Cockerill et al., 1995), this angiogenesis process takes place without the addition of any exogenous modulator like growth factors (except during the first 10 days of culture) or tumor promoting agents. This phenomenon has been attributed to the synthesis of angiogenic molecules by endothelial cells and fibroblasts, and the presence of a complex extracellular matrix synthesized by the fibroblasts (Berthod et al., 1996). We previously demonstrated that capillary-like structures (CLS) were only composed of endothelial cells that deposited basement membrane proteins (laminin and type IV collagen) on their basal side. These tubular structures featured a lumen the entire length of the CLS and were organized in a network of branching tubes that reached the bottommost portion of the dermis (Hudon et al., 2003).

In this study, we demonstrated that the ERCT, made of HUVEC or human microvascular endothelial cells (HMVEC), was a powerful model to finely analyze, quantitatively and qualitatively, the influence of angiostatic drugs on angiogenesis.

Materials and methods

Human cells isolation

Human fibroblasts were isolated from human skin biopsies after breast reductive surgeries using 0.2 IU/ml collagenase H (Boehringer Mannheim, Montreal, Canada). Cells were grown in DMEM (Dulbecco-Vogt modification of Eagle's medium) (Invitrogen, Burlington, Canada) supplemented with 10% Fetal Calf Serum (FCS) (HyClone, Logan, Utah, USA), 100 U/ml penicillin (Sigma, Oakville, Canada) and 25 µg/ml Gentamicin (Schering, Pointe-Claire, Canada) in 8% CO_2 at 37ºC. The medium was changed three times a week.

Human umbilical vein endothelial cells (HUVEC) were obtained from healthy newborns by enzymatic digestion with 0.250 µg/ml thermolysin (Sigma). Veins were cannulated at both ends and washed with calcium-free HEPES solution (ICN, St-Laurent, Canada). A thermolysin solution was then injected to rinse and fill the vein and the cord was placed in calcium-free HEPES solution at 37ºC. After 30 min incubation, the veins were perfused with M199 medium (Sigma) containing 10% FetalClone II serum (HyClone) and antibiotics (100 U/ml of penicillin G, and 25 µg/ml of gentamicin) (Black et al., 1998).

HUVEC were centrifuged and resuspended in M199 medium supplemented with 20% FetalClone II serum (Hyclone), 2.28 mM glutamine, 40 IU/ml heparin (Leo Laboratories, Pickering, Canada), 20 µg/ml endothelial cell growth supplement (Sigma) and antibiotics (100 U/ml of penicillin G and 25 µg/ml of gentamicin). HUVEC were plated on gelatin-coated tissue culture flasks and characterization was assessed as previously described (Black et al., 1998).

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Human microvascular endothelial cells (HMVEC) were purified from the foreskin of a healthy baby using Dynabeads CD31 (Dynal, Lake Success, NY, USA) (Richard et al., 1998). Briefly, pieces of skin tissue were incubated overnight in a Hank's balanced salt solution (Sigma) containing 2% fetal calf serum (HyClone) and 1% dispase II (Roche Diagnostics, Laval, Canada). The epidermis was removed and endothelial cells were extruded by pressing the dermal layer. Mixed cells were centrifuged, resuspended in EGM-2 medium (Clonetics, San Diego, CA, USA) and then plated on gelatin-coated tissue culture flasks for one week. Cells were then removed from the culture flask by trypsin/EDTA (ICN) and incubated with Dynabeads CD31 for 30 minutes. HMVEC characterization was assessed as previously described (L'Heureux et al., 1993).

Collagen/ glycosaminoglycan/chitosan biopolymer preparation

Collagen-glycosaminoglycan-chitosan biopolymers were prepared using a technique previously described (Berthod et al., 1993). These sponges were prepared with type I, III bovine collagen (Laboratoire Perouse Implant, Chaponost, France), chitosan (95% deacetylated (SADUC, Lyon, France) and chondroitin 4-6 sulfates (SADUC, Lyon, France) which were dissolved in 0.1% acetic acid. After mixing, 1 ml/well (3.8 cm^2) of the final solution was poured into 12 well plates (Becton Dickinson, Toronto, Canada) and frozen overnight at –80°C. The frozen plates were then lyophilized in a Genesis 12EL vacuum freeze-dryer (Virtis, Gardiner, N.Y., USA).

Endothelialized reconstructed dermis preparation

The ERCT was prepared with a suspension of 1:1 ratio of human fibroblasts and HUVEC or HMVEC. A volume of 3 ml was seeded on the top of a chitosan-glycosaminoglycan-collagen sponge at a final concentration of $2.1x10^5$ cells/cm^2 (Black et al., 1998). The cells were cultured under submerged conditions and fed with 3 ml of EGM-2 medium (Cambrex, Baltimore, MD)

supplemented with 50 µg/ml ascorbic acid (Sigma). After 10 days, ERCT were cultured in DMEM-Ham's (Invitrogen), supplemented with 10% fetal bovine serum, 0.4 µg/ml hydrocortisone (Calbiochem, LaJolla, CA, USA), 5 µg/ml bovine insulin (Sigma), 100 µg/ml ascorbic acid and antibiotics. Seven days later, the ERCT were elevated at the air-liquid interface and cultured in the same medium for 2 additional weeks.

ERCT treatment with drugs

The angiogenesis-inducing agent vascular endothelial growth factor (VEGF; Sigma) or inhibiting agents, Tamoxifen (Sigma), Ilomastat (Chemicon, Temecula, CA, USA) and Echistatin (Sigma) were added three times in the medium for a total period of 7 days. In the first experiment drugs were added between days 17 and 24 (three successive doses on days 17, 19 and 22) at the following concentrations: VEGF (15, 30, 45 and 60 ng/ml), Tamoxifen (10, 20, 30 and 40 µM), Ilomastat (12.5, 25, 50 and 75 µM) and Echistatin (0.03, 0.06, 0.09 and 0.12 µM) to assess their dose-response effect. In a second experiment, a unique concentration of VEGF (15 ng/ml), Tamoxifen (40 µM), Ilomastat (50 µM) and Echistatin (0.06 µM) was added between days 10 and 17 to analyze the long-term effect of each drug on HUVEC-ERCT, compared to HMVEC-ERCT. The analyses were performed on biopsies taken after 10, 17, 24 and 31 days of treatment. Results obtained with Ilomastat were compared with a control treated with 30 µl/ml DMSO.

ERCT treated under a condition of hypoxia

HUVEC-ERCT were cultured for 24 days and then incubated 16 h in an anaerobic jar (VWR, Montreal, Canada) containing a disposable carbon dioxide/hydrogen gas-generating system (Anaerocult A™, VWR) which induced a deprivation of oxygen by the supplementation of atmosphere with CO_2. The total deprivation of oxygen was assessed with a test strip

(Anaerotest™, VWR). The histological analyses were performed on ERCT biopsies taken 1, 4, 7 and 10 days after the 16h hypoxia treatment.

Immunohistochemistry

Frozen 4 μm sections were incubated in a blocking solution containing phosphate-buffered saline (PBS) and 1% (w/v) bovine serum albumin (BSA) (Sigma). Fluorescent staining was performed by subsequent incubations with a primary mouse monoclonal anti-human Platelet-Endothelial Cellular Adhesion Molecule-1 (PECAM-1) antibody (Chemicon) and a Rhodamine-conjugated goat anti-mouse IgG-IgM (Chemicon) secondary antibody, mixed with Hoechst (1/100) (Sigma) to stain the cell nuclei. Sections were examined using a Nikon Eclipse E600 fluorescence microscope.

Quantification and evaluation of areas containing capillary-like structures

Samples were fixed with Histochoice's solution (Armesco, Solon, Ohio, USA), dehydrated and embedded in paraffin. Six microns sections were cut, stained with Masson's trichrome and visualized on a Nikon Eclipse E600 fluorescence microscope. The area of each capillary-like structure (CLS) was calculated on digital microphotographies using Metaview software (Universal Imaging Corporation, Downingtown, PA, USA). The number of CLS was measured with the NIH image version 1.62 software (NIH, Bethesda, MA, USA). Four samples (with the mean of two slides per sample) were analyzed for each group for both the number and surface area of CLS. The data obtained were analyzed by the two-way ANOVA test, and $p < 0.05$ was considered as significant.

Results

To demonstrate the efficacy of the ERCT in discriminating the angiostatic potential of molecules, drugs were added in the culture medium between day 10 and 17, and biopsies were taken after 10, 17, 24 and 31 days of culture. One week after the removal of 15 ng/ml VEGF, the number of CLS in ERCT made of HMVEC (HMVEC-ERCT) was significantly higher than in the control, as observed on histological cross-sections and by immunohistochemistry using an antibody specific to PECAM-1 (figure 1g versus 1c), and after counting of CLS on histological cross-sections (*$p<0.05$) (figure 2). Similar results were not observed with ERCT made of HUVEC (HUVEC-ERCT) (figure 1e,f versus 1a,b), although two times more capillary-like tubes were found with HUVEC-ERCT (Figure 2). Both the HUVEC and HMVEC-ERCT showed much fewer CLS than the control when treated with Tamoxifen (40 µM) from day 24 to day 31 (figure 2). This difference was clearly visible by histology and immunohistochemistry (figure 1i-l), along with a likely decrease in the total number of cells (stained with nucleus specific marker Hoechst) and of extracellular matrix (collagen in blue on histology) that could account for a general toxicity rather than a specific angiostatic effect. However, this toxic effect was only visually suspected and has not been further investigated. Echistatin (0.06 µM) induced a significant decrease in the number of CLS from day 17 to day 31 in HUVEC-ERCT, and from day 17 to day 24 in HMVEC-ERCT (Figure 2). No difference was observed with Ilomastat (50 µM), except a slight decrease at day 17 in HUVEC-ERCT (figure 2). Tamoxifen induced a drastic and continuous decrease in the number of CLS even 2 weeks after its removal in both ERCT models. Echistatin induced a decrease in the number of CLS, without evident signs of toxicity, which

appeared to be reversible 2 weeks after drug removal in the HMVEC-ERCT. Ilomastat did not show an effective angiostatic effect in this set of experiments.

Experiments with HUVEC-ERCT were performed to evaluate a dose-response effect with increasing concentrations of drugs added in the culture medium between days 17 and 24, which is the period of maximal CLS formation.

When the ERCT was treated with VEGF (15 to 60 ng/ml) (figure 3), a significant increase of the number of CLS was observed (*$p < 0.05$, ** $p < 0.005$, n=4), compared to the control, after drug removal at 45 ng/ml. Seven days after drug removal, this effect was also observed at 30 ng/ml.

Tamoxifen (10 to 40 µM) (figure 3) induced an increase in the number of CLS at 10 µM, 7 days after drug removal. No significant difference was obtained at 20 µM, although a significant decrease was measured at 30 and 40 µM. Since the number of CLS at 40 µM remained unchanged between day 24 and day 31, we concluded that Tamoxifen completely blocked capillary-like formation after drug removal, but did not alter previously formed CLS. This inhibition was not reversible at 40 µM one week after drug removal, probably because of a toxic effect of the drug. Tamoxifen had no significant effect just after drug removal.

When Echistatin (0.03 to 0.12 µM) (figure 3) was added to the culture medium, a diminution of the number of CLS at 0.06, 0.09 and 0.12 µM was observed, although this effect was only maintained at the 0.12 µM concentration during the 7 days following the removal of the drug. Thus, Echistatin has a moderate angiostatic effect early after its addition, but this effect was rapidly reversible after drug removal.

The addition of Illomastat (12.5 to 75 µM) (figure 3) induced a significant decrease in the number of CLS at 50 ($p < 0.05$) and 75 µM ($p < 0.005$) after drug removal. This angiostatic effect was observed at all concentrations 7 days after drug removal ($p < 0.005$). At 75 µM, a 4.5 times decrease in the number of CLS was observed compared to the control. This inhibition of

angiogenesis was abolished after drug removal, since at day 31, more CLS were observed at all concentrations compared with day 24. Ilomastat induced a very robust and very precocious inhibitory effect on CLS formation, since at 75 µM, nearly no additional CLS was formed following the addition of the drug (the number of CLS at day 17 and day 24 was similar at this concentration; data not shown).

The ERCT allowed us to analyze the modulation of hypoxia on CLS formation. The *in vitro* model was placed for 16 h in an anaerobic jar and histological analyses were performed on biopsies taken 1, 4, 7 and 10 days after the treatment. One week after hypoxia (figure 4b, c and d), no significant difference in the number of CLS (figure 4e) was detected compared with control (figure 4a), but the number of very large CLS (over 300 μm^2) was 10 elevated times compared to the control (*$p < 0.05$, $n=3$) (figure 4f).

Discussion

The aim of this work was to analyze, quantitatively and qualitatively, the influence of 3 anti-angiogenic molecules on our model, in order to demonstrate that it is highly suitable for determining the angiostatic potential of drugs. VEGF was used as a positive control of angiogenesis and Tamoxifen, Ilomastat and Echistatin were chosen as well known angiostatic compounds, which block different and major steps of the angiogenesis process.

VEGF, which is known to have a strong angiogenic effect (Ferrara, 2004) induced an augmentation in the number of CLS in the ERCT at 45 ng/ml after the end of a 7 day treatment. Seven days after the removal of VEGF, a significant increase was observed at both 30 ($p < 0.05$) and 45 ng/ml (0.005). The 30 ng/ml concentration needed a one week time period after treatment to induce an increase in the CLS number. This showed that the induction of the VEGF-mediated angiogenesis was a slow process at lower concentrations. However, at 60 ng/ml, the angiogenic drug didn't show a significant augmentation. This could be explained by an increased competition of the molecule with its receptor and by the fact that VEGF is also known to affect the intussusception (non-sprouting angiogenesis) (Ferrara, 2001).

Tamoxifen was shown to have concurrent pro and anti-angiogenic actions when it interacts with estrogen receptors (Lee et al., 2004). However, this mediation through the estrogen receptor can not account for all of the Tamoxifen effects (Reddel et al., 1985). Tamoxifen is also known to inhibit angiogenesis *in vitro* and *in vivo* by the inhibition of endothelial cell proliferation (Gagliardi et al., 1996). This effect could be induced by blocking ornithine decarboxylase, cholesterol synthesis, microsomal lipid peroxidation, or cyclins, but predominantly by blocking the calcium dependent protein kinase C (Gagliardi et al., 1996). In contrast, Tamoxifen

demonstrates an angiogenic effect by stimulating the expression of VEGF (Hyder et al., 1997), TGF-β (Grainger et al., 1993), and by down-regulating CD36 expression (Silva et al., 1997).

Our model depicted these complex processes very well. In the ERCT, an angiogenic effect of Tamoxifen was observed at 10 µM, which could be explained at least in part by the stimulation of VEGF expression by fibroblasts. A concentration dependant anti-angiogenic effect was detected starting at 20 µM. This effect became statistically significant one week after the drug removal at 30 µM, and was clearly cytotoxic at 40 µM. The lag time needed before seeing a decrease in the CLS number could be explained by the delay necessary for Tamoxifen metabolites to reach the critical concentration for inducing an irreversible modification of protein kinase C (McNamara et al., 2001). However, this decrease should not be specific to an angiogenesis inhibition since the total number of cells seemed to be notably decreased (see number of blue nuclei in figure 1I compared to 1A).

Echistatin is well known to inhibit adhesion, migration and survival of human umbilical cord vein endothelial cells (Juliano et al., 1996). This effect is induced by its binding to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin on endothelial cells and are two integrins that participate together in different angiogenic processes like proliferation, migration and survival (Alimenti et al., 2004). Echistatin induces a considerable dose-dependent decrease in the number of CLS, as observed in the literature with other antagonists of integrin $\alpha_v\beta_3$ (Brooks et al., 1994). This inhibition effect was still detectable at the highest dose 7 days after drug removal, but was abolished at 0.06 µM and at 0.09 µM. This prolonged effect at 0.12 µM could be explained by the non-covalent irreversible binding of Echistatin to $\alpha_v\beta_3$, which ultimately induces apoptosis in endothelial cells (Alimenti et al., 2004). At lower doses, only a transient effect was observed and is probably due to internalization of blocked integrins which never reach the critical level that activate apoptosis.

Ilomastat is known to inhibit angiogenesis by blocking some matrix metalloproteinases (MMP) such as the gelatinases MMP-2 and MMP-9 (Galardy et al., 1994). Both are specialized in the degradation of different basement membrane components and seem to modulate endothelial cell attachment, proliferation and migration during angiogenesis (Stetler-Stevenson, 1999). The angiostatic effect obtained with Ilomastat hinged highly upon the time it was added to the ERCT culture. If added between days 10 and 17, Ilomastat had no clear inhibitory effect on CLS formation (Figure 2). In contrast, Ilomastat added to the ERCT culture between days 17 and 24 induced a dose-response diminution in the number of CLS at all concentrations 7 days after drug removal, as observed in other angiogenesis models (Galardy et al., 1994). This inhibition of CLS formation was drastic (3 times less capillaries compared to control at 75 µM) and took effect rapidly after drug addition in the culture medium, since the effect was already achieved at the end of the drug incubation period. However, one week after the drug removal, a two fold increase of the number of CLS was observed at 75 µM, indicating that the inhibitory effect of the drug was abolished immediately after drug removal, as compared with the two fold increase in CLS number in controls during the same period. Indeed, Illomastat has a half-life of 13 minutes and necessitates a continuous treatment to maintain its inhibitory effect on the MMP synthesis or secretion (Galardy et al., 1994). Since the addition of Ilomastat between days 10 and 17 which is a period of low CLS formation, did not induced a significant decrease in CLS formation compared to its addition between days 17 and 24, this result highlights the critical importance on the kinetic timing of drug addition during the CLS formation.

Finally, Ilomastat appeared to be the most powerful angiostatic drug since it rapidly and completely inhibits CLS formation at 75 µM, while at the same time it did not seem to induce a toxic effect on cells.

Since the local oxygen tension is well known to have a profound effect on the vasculature by compensating the vascular insufficiency through the induction of angiogenesis (Knighton et al., 1983) , we investigated the modulation of hypoxia on the CLS in the ERCT. We demonstrated that hypoxia can modulate the shape of CLS formed in the ERCT. We observed a 10 fold increase in the formation of CLS larger than 300 µm in diameter, although the total number of CLS remained unchanged. This effect could be explain by a hypoxia-mediated VEGF expression which might induce non-sprouting angiogenesis (Risau, 1997). An increase in the capillary diameter of cardiac tissue has also been described under conditions of oxygen deprivation in fetal sheep (Martin et al., 1998). Thus, the ERCT allowed us to detect an unexpected modulation of the capillary-like network by hypoxia, which gives evidence of the powerfulness of this model in discriminating subtle modulations of different parameters in the angiogenesis process.

Most *in vitro* studies performed on endothelial cell activation utilize cells isolated from human umbilical cord veins. However, it is well-known in the literature that endothelial cells are greatly heterogeneous and also depend on the tissue from which they originated (Garlanda and Dejana, 1997). To assess whether similar results could be obtained with microvascular endothelial cells cultured in our model, we have compared the effect of the drugs on CLS produced with endothelial cells isolated from large vessel (HUVEC) versus CLS made of endothelial cells from microvascular endothelium (HMVEC). The two endothelial cell types showed similar results except in the case of Ilomastat, where the HMVEC-ERCT featured two times less CLS number compared with the HUVEC-ERCT (Figure 1, 2).

By using 3 angiostatic compounds which inhibited 3 major components of the angiogenic process, i.e. endothelial cell proliferation (Tamoxifen), endothelial cell migration and apoptosis control via integrins (Echistatin) and MMP function (Ilomastat), we demonstrated the strength of our model to accurately discriminate the angiostatic potential of drugs. One of the major

advantage of the ERCT is that it can be maintained in culture for a long period (over 30 days), in contrast with the other models in which CLS are produced in a few hours in response to a boost of growth factors and are degenerated in less than 72 h (Troyanovsky et al., 2001). With the ERCT, the moment and the duration of the treatment can be chosen over at least a 31 day period, in order to observe an inhibition in the CLS development (earlier treatment) or a regression of pre-existing vessels (later treatment). In addition, this culture period gives the possibility to analyze the long-term effect of the drugs on angiogenesis, as we have demonstrated that some molecules (such as Echistatin and Ilomastat) can induce a rapid, but transient decrease on CLS formation while others have a delayed inhibitory effect on angiogenesis, as is the case with Tamoxifen. A toxic effect of the molecule can also be suspected by observation of histological tissue morphology, and in turn quantified by cell extraction and counting, or by the use of viability tests such as a MTT test. Finally, the analysis of CLS morphology also allows the control of the CLS size.

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Footnotes

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Legends for Figures

Figure 1. Immunohistochemical and histological characterization of CLS formed by HUVEC or HMVEC after 31 days of culture in the presence of pro or anti-angiogenic agents in the medium between days 10 and 17.

Cryopreserved sections were immunostained with Hoechst (blue) for visualization of cell nuclei and an antibody directed against human PECAM-1 (red), specific to endothelial cells (a, c, e, g, i, k, m, o, q and s) and paraffin-embedded sections were stained with Masson's trichrome (b, d, f, h, j, l, n, p, r and t). When the ERCT, made with HUVEC (a, b, e, f, i, j, m, n, q and r), was treated with the angiogenic factor VEGF (15 ng/ml) (e, f), it showed more CLS (arrows in B) compared to the control (A, B). However, when the ERCT was treated with Tamoxifen (40 µM) (i, j), Echistatin (0.06 µM) (m, n), or Ilomastat (50 µM) (q, r), a decrease in the number of CLS was observed, compared to the control. In addition, a drastic reduction in the number of cells (nuclei stained in blue for i, compared with a) and the amount of extracellular matrix was clearly visible with Tamoxifen treatment (i, j), compared to control (a, b), suggesting a toxic effect of this drug at 40 µM. Similar results were obtained with HMVEC-made ERCT (c, d, g, h, k, l, o, p, s and t), when treated with VEGF (g, h) and Tamoxifen (k, l) compared to the control (c,d). In contrast, no clear difference in the number of CLS was observed with Echistatin (o, p) and Ilomastat (s, t). Furthermore, the total number of CLS observed in HMVEC-made ERCT was always clearly lower compared to ERCT made with HUVEC. Scale bar in a: 200 µm.

Figure 2. Drug response of HUVEC compared to HMVEC-made ERCT.

These analyses were performed on biopsies taken after 10, 17, 24 and 31 days of treatment when drugs were added between day 10 and 17. For the HUVEC-ERCT, no significant difference in the number of CLS was detected with 15 ng/ml of VEGF at all time points. In contrast, a significant increase was observed from day 17 to day 31 in the HMVEC-ERCT. Echistatin (0.06 µM), Tamoxifen (40 µM) and Ilomastat (50 µM) induced a significant reduction (*$p < 0.05$ and **$p < 0.005$, n=4) in the number of CLS at days 17, 24 and 31, day 24 and 31, and days 17, respectively, compared to the control. For the HMVEC, similar results were obtained Echistatin (except at day 31) and Tamoxifen, but no effect was observed with Ilomastat at any time points. Finally, at least two times less CLS were observed at all time points in HMVEC-ERCT, compared with HUVEC-ERCT.

Figure 3. Evaluation of the effect of increasing concentrations of drugs on CLS formation in the HUVEC-ERCT.

Drugs were added in the culture medium between days 17 and 24. These analyses were performed on biopsies taken immediately after the end of drug treatment (day 24) and one week after the removal of drugs (day 31). When the ERCT was treated with 30 and 45 ng/ml of VEGF, a significant increase in the number of CLS was observed, compared to the control (*$p < 0.05$, **$p < 0.005$, n=4). Furthermore, the VEGF treated group induced a decrease in the number of CLS at 60 ng/ml. Tamoxifen (10 to 40 µM), Echistatin (0.03 to 0.12 µM) and Ilomastat (12.5 to 75 µM) induced a significant decrease in the number of CLS. Tamoxifen was angiogenic at 10 µM but angiostatic at 30 and 40 µM, 7 days after drug removal. It completely inhibited the formation of CLS between day 24 and 31 at 40 µM. Echistatin induced an inhibitory effect on angiogenesis at 0.06 to 0.12 µM, although this effect was abolished one week after the drug

removal (except at 0.12 µM). Ilomastat induced a major decrease in CLS formation and was reported until day 31. The grey line indicates the number of CLS in the control after 24 days of culture.

Figure 4. Effect of Hypoxia on CLS formation in the HUVEC-ERCT.

These analyses were performed on biopsies taken 1, 4, 7 and 10 days after the ERCT were placed in a hypoxia environment for 16 hours. Larger CLS were observed in the ERCT on histological cross-section (b, arrows), as compared to the control (a, arrows), one week after the treatment. The quantitative analysis of the CLS area (d) demonstrated a 10 fold increase of tubules larger than 300 µm, compared with the control (*$p < 0.03$, n=3), but no difference in the number of CLS between hypoxic and normoxic samples was observed (c) (n=4). Scale bar in A: 200 µm.

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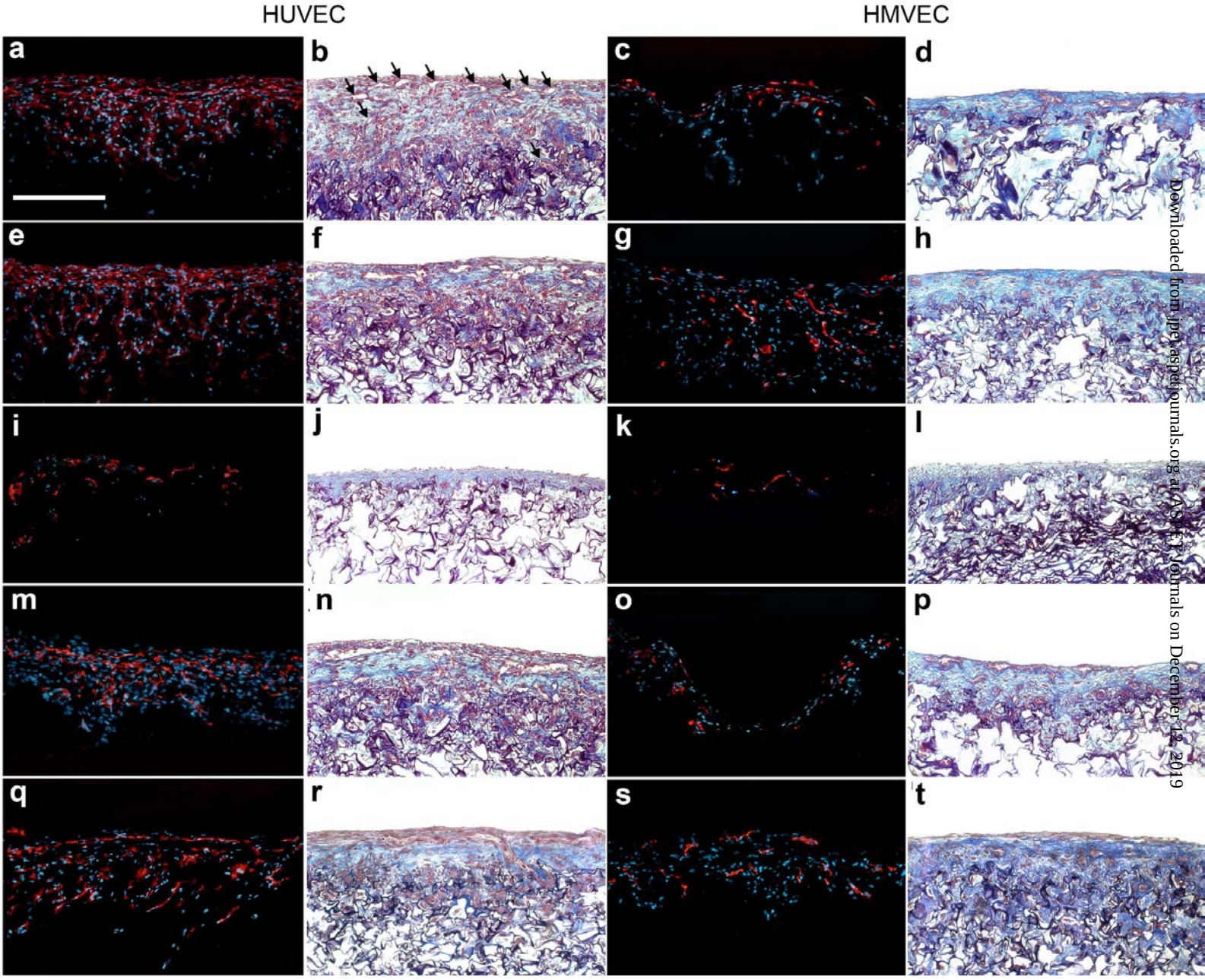

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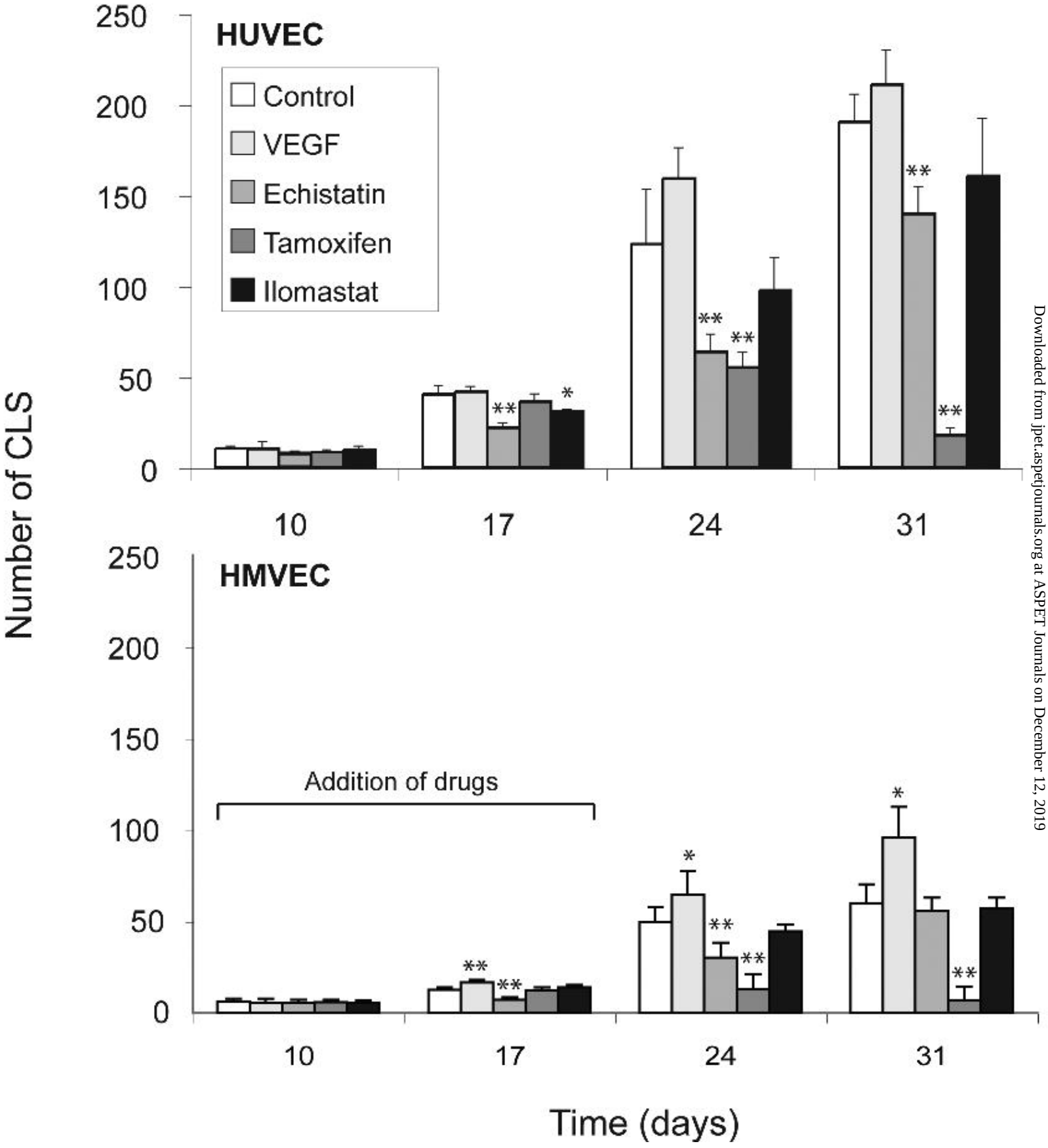

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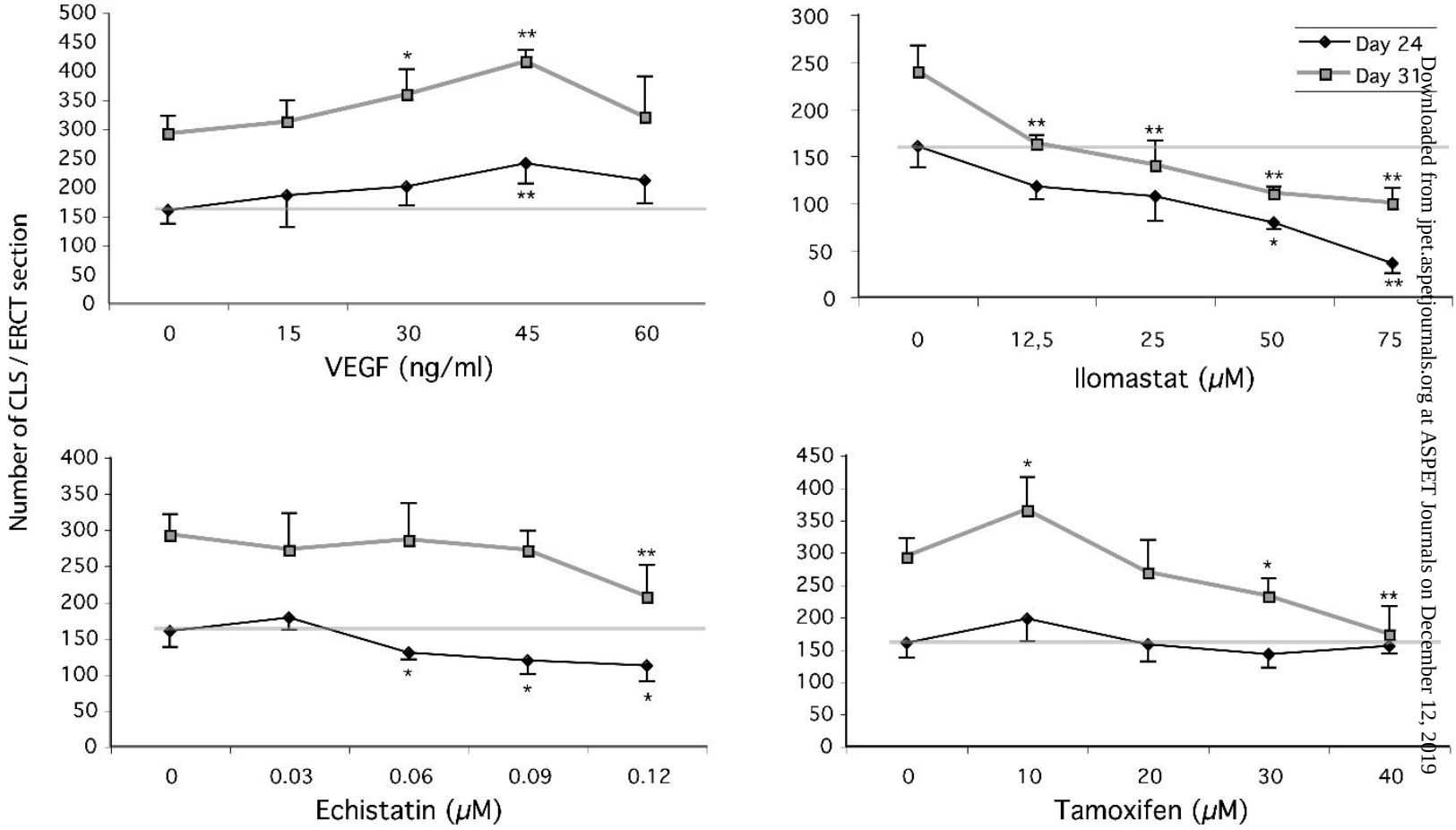

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