Growth Inhibiting Effects of Progesterone, 17β-Estradiol and 2-Methoxyestradiol on Human Endothelial Cells

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Abstract

Objective: Angiogenesis is the formation of new capillary blood vessels by sprouting from existing microvasculature. It is critical for a variety of pathological and non-pathological processes in vertebrates. Steroids and their metabolites have been shown to inhibit angiogenesis and promote morphogenesis in a variety of tissues.
Materials and Methods: Immunocytochemistry was used to stain the cells with various antibodies. The IC50 concentration was determined using a coulter counter and flow cytometry was used to analyze the externalization of phosphatidylserine as an early event in the apoptotic pathway.
Results: We found that the steroids progesterone and 17β-estradiols as well as 2-methoxyestradiol had strong proliferation inhibiting properties on different primary cultures of endothelial cells. The growth inhibiting effect of 2-methoxyestradiol occurs in confluent and in bFGF-induced proliferating cultures of endothelial cells at similar concentrations. In proliferating cultures of endothelial cells this effect occurs not due to cytotoxicity but seems to be mediated by an apoptotic mechanism as shown by FACS analysis.
Conclusion: Our results show that the growth inhibiting effect of 2-Me in human endothelial cells occurs by an apoptotic mechanism. Moreover it demonstrates that the antiproliferative effects of 2-Me are independent of the proliferation status of endothelial cells.

Keywords: estradiol, progesterone, 2-methoxyestradiol, endothelial cells, apoptosis

Özet

Progesteron, 17β-estradiol ve 2-metoksiestradiolün İnsan Endotelyal Hücrelerdeki Büyümeyi İnhibe Edici Etkisi

Amaç: Anjiyogenez mevcut mikro damar yapısından yeni kapiller kan damarı oluşumudur. Bu olay omurgalılarda çeşitli patolojik ve patolojik olmayan süreçler açısından önemlidir. Estradiol ve bazı metabolitlerinin anjiyogenezi engellediği ve çeşitli dokularda morfogenezi indüklediği gösterilmiştir.
Materyal ve Metot: Hücreleri boyamak için çeşitli antikorlar ile immün histokimya kullanılmıştır. Coulter counter kullanılarak IC50 konsantrasyonları belirlendi ve Flow cytometry ile apoptotik yolun erken bir bulgusu olan fosfatidilserin ayrışması analiz edildi.
Sonuçlar: Steroidler; progesteron, 17-estradioller ve hatta 2-metoksiestradiolün çeşitli primer endotelyal hücre kültürleri üzerinde kuvvetli proliferasyonu inhibe edici etkisi olduğu tespit edildi. 2-metoksiestradiolün büyümeyi inhibe edici etkisi devamlı ve bFGF ile proliferasyonu indüklenmiş endotelyal hücre kültürlerinde benzer oranda gerçekleşmektedir. Prolifere olan endotelyal hücre kültürlerindeki bu etki sitotoksisiteye bağlı değil, fakat FACS analizi ile gösterildiği gibi muhtemelen apoptotik mekanizmalar ile gerçekleşmektedir.
Tartışma: Sonuçlarımız 2-metoksiestradiolün insan endotelyal hücreleri üzerindeki büyümeyi inhibe edici etkisinin bir apoptotik mekanizma ile ortaya çıktığını göstermektedir. Ayrıca 2-metoksiestradiolün antiproliferatif etkisinin endotelyal hücre proliferasyonundan bağımsız gerçekleştiği görülmektedir.

Anahtar sözcükler: estradiol, progesteron, metabolitler, endotel hücreleri, apopitoz

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Introduction

Angiogenesis is the development of new blood vessels through the process of endothelial cell proliferation and outgrowth from preexisting vessels. It is regulated by a complex series of growth factor interactions and hormones, including stimulatory, modulatory and inhibitory regulators and is associated with changes in the extracellular matrix required to allow migration of the newly formed vessels. Steroid hormones like estradiols, progesterone and corticoids are well known modulators of cellular functions. They act via transcription factors as monomers, homodimers as well as by crosstalk to other signaling pathways. The effects of nuclear receptors are mediated through recruitment of co-regulators. Besides their effects on gene transcription steroid hormones may transduce signals by interacting with compounds of the cellular membranes e.g. Phospholipase C (PLC) and subsequent stimulation of the protein kinase C (PKC) pathways (summarised in [1]) and activation of voltage gated Ca^{2+} channel opening [2].

2-Methoxyestradiol (2-Me) is a physiological metabolite of estradiol, which is excreted with the urine. It was demonstrated that 2-Me had strong antiproliferative effects on MCF-7, HeLa and other human cells [3-6]. Additionally, it was suggested that estradiol metabolites inhibit tumor growth by different mechanisms, such as inhibition of the proliferation of endothelial cells [3,7,8], induction of apoptosis by p53 dependent and independent actions [9-11] and cell arrest in G2/M-phase by inhibiting tubulin polymerization [12]. Recently, it was demonstrated that angiogenesis is efficiently blocked by 2-Me in malignant tumors [6,13,14]. As 2-Me has a negligible affinity to the estradiol receptor [15-17] it seems plausible to suggest that some of the growth inhibiting effects of 2-Me are exerted by a non receptor mediated mechanism of action [18,19].

To understand in more detail how gonadal steroids and their metabolites influence the growth of endothelial cells we studied the proliferation behavior of human umbilical vein endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs), human dermal microvascular endothelial cells (HDMVECs) after incubation with steroid hormones 2-methoxyoestradiol, 17β-estradiol and progesterone.

Materials and Methods

Cell Cultures and Reagents

Human endothelial cells were isolated by a method adapted from Jaffe and coworkers (38). Preparations of umbilical cords were performed no later than 24 hours post partum. The vein and artery were cannulated and rinsed with Dulbecco's phosphate buffered saline (PBS; Gibco, Germany) in order to remove blood cells. Subsequently, the vessel was filled with 0.6 units Dispase II solution (Roche Diagnostics, Germany) and incubated for 40 minutes at 37°C. After incubation the cord was gently kneaded to release the endothelial cells. Finally the veins and arteries were drained and washed with PBS. After centrifugation for 5 min at 1000 rpm, the cells were resuspended in endothelial growth medium (EGM, PromoCell, Germany) supplemented with 3% fetal calf serum (FCS, Gibco, Germany) and cultured in gelatine-coated (1.5% in water) culture flasks at 37°C and 5% CO_2. Vital cells were identified by staining with tryptan blue solution 0.5% in 1xTrypsin EDTA solution. The identity and homogeneity of the isolated endothelial cells were determined by FACS analysis using monoclonal antibodies directed against VEGFR-2/KDR, TIE2 and CD31 and by measuring [H^3] thymidine incorporation as a marker for DNA synthesis after stimulation by the endothelial cell specific mitogen VEGF. The expression of progesterone and estradiol receptors were determined by immunocytochemistry using monoclonal antibodies directed against the human progesterone and estradiol receptors (both from DAKO Glostrup, Germany). The MCF-7 cell line (ATCC, Rockville, USA) was cultured at 37°C in MEM alpha medium (Gibco, Germany) 10% FCS, 0.5% Insulin (Sigma, Germany) and 5% CO_2.

This study was approved by the internal review board at the University of Freiburg, Faculty of Medicine.

Flow Cytometry

Endothelial cells were transferred to 96-well cluster plates ($1x10^5$ cells/well) and pelleted by centrifugation at 1200 rpm for 2 min. Cells were resuspended in 50 µl HBSS/2% FCS containing 1µg/well mouse anti human CD 31 antibody (Dianova, Germany), mouse anti human KDR antibody and mouse anti human Tie2 antibody (both were generously provided by P. Reusch, Institute for Tumor Biology, Freiburg and described previously [20,21]), respectively. After a 30 min incubation at 4°C, primary antibody was removed, cells were washed twice and placed in FITC-conjugated, anti-mouse secondary antibody (Sigma, Germany) for 30 min at 4°C in the dark. Secondary antibody was removed, cells were washed and incubated in HBSS/2% FCS containing propidium iodide (PI) for 3 min at 4°C. After removal of PI, cells were resuspended in 400 µl HBSS/2% FCS. Flow cytometry was done with a Becton Dickinson FACSort. The scan area was set to encompass the available culture area for each of the chambers. Data acquisition/analysis parameters were set to include only single cells and to exclude debris and aggregates.

Endothelial Cell Proliferation Assays

To test the inhibitory potency of gonadal steroid hormones on endothelial cell proliferation, HUVECs, HUAECs, HDMVECs were seeded in 24 well-plates containing $1x10^4$ cells under confluent conditions. Under proliferating conditions $5x10^3$ (HUAECs) or $7x10^3$ (HDMVECs, HUVECs) were seeded. Subsequently cells were incubated in an appropriate medium for 24 hours without steroid hormones and stimulated for 6 days with steroid hormones Progesterone, Methoxyestradiol, 17β-estradiol which were purchased by Sigma, Deisenhofen, Germany and diluted in an appropriate volume of Ethanol and 5 ng/ml bFGF (PromoCell, Germany) including medium change every second day. Afterwards

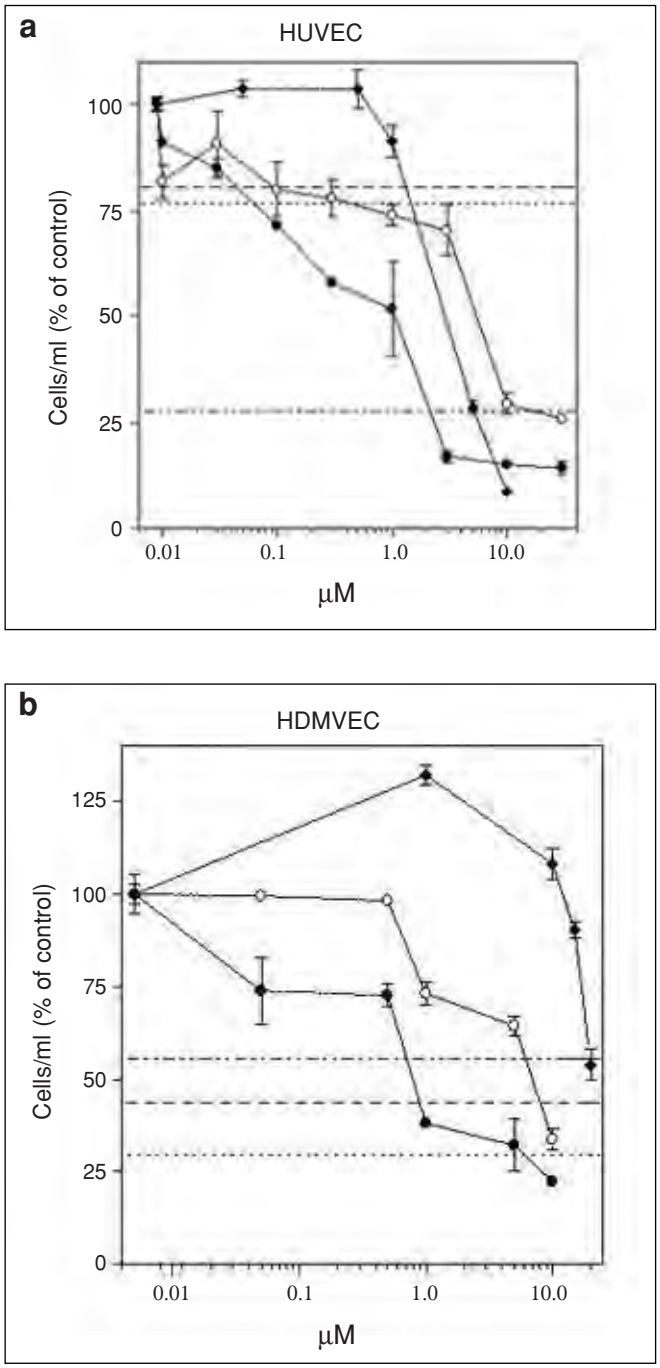
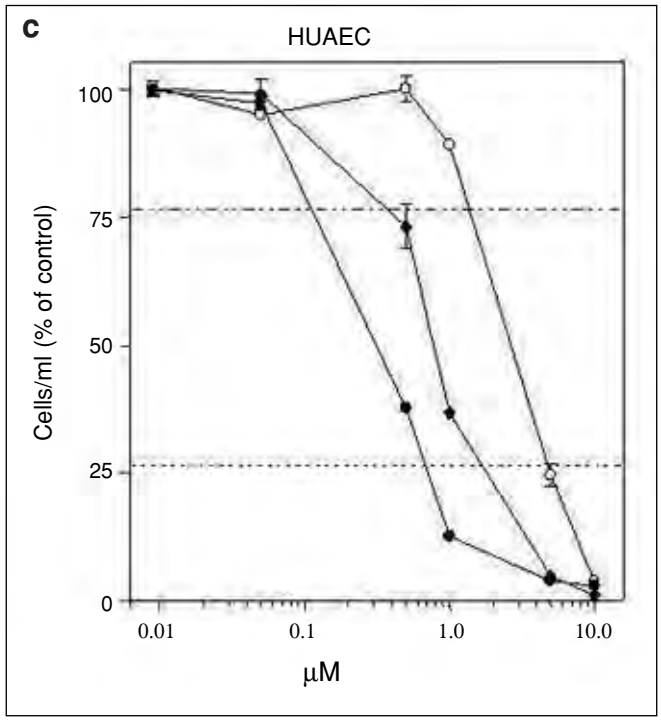


Figure 1. Proliferation assay of HUVECs (A), HDMVECs (B) and HUAECs (C) after stimulation with bFGF and various gonadal steroid hormones. 17β-estradiol (o), 2-methoxyestradiol (●), progesterone (◆). The number of cells without stimulation which are used as control cells are presented as dotted lines:
17β-estradiol: .....
2-methoxyestradiol: ----------
progesterone: ——————

cells were washed twice with PBS and, after trypsination, cell number per well was determined in a Coulter counter (Coulter-electronics, Krefeld, Germany) and compared to untreated cells. To determine whether the observed effects of steroid hormones on HUVECs were reversible, we incubated the cells for 7 days with respective hormones under conditi-



ons described above. In half of the cells seeded, the medium was changed to steroid free medium after 4 days. After a regeneration phase of 3 days, the amount of viable cells was determined in a Coulter counter (Coulter-electronics, Krefeld, Germany).

Immunocytochemistry

Cells were seeded in silanized chamber slides (Greiner, Frickenhausen, Germany) and cultivated for 24 h. Afterwards cells were fixed with methanol/acetone (-20°C). After washing with phosphate-buffered saline, cells were incubated for 45 min with H_2O_2 (0.3% in PBS) to block internal peroxidases. Then, cells were incubated for 26 h at 4°C temperature with mouse monoclonal anti-estradiol or progesterone antibody (DAKO, Glostrup, Germany). Bound estradiol or progesterone receptor antibody was detected with biotin-conjugated rabbit-anti-mouse antibody (Sigma, Deisenhofen, Germany). For the staining of marked estradiol and progesterone receptors, the Vectastain kit (Vector Laboratories Burlingame) was used according to the manufacturers' instruction.

Statistics

Results were expressed as mean ± standard error of means (SEM). A p-value <0.05 were considered as statistically significant. Repeated measures were analyzed using two way analysis of variance (ANOVA). Unpaired data were analyzed using students t-test.

Results

Several studies suggest that normal as well as pathological vessel formation is associated with an effect of estradiols and estradiol metabolites on endothelial cell angiogenic activity [3,22]. To analyze the effects of estradiols and their metabo-

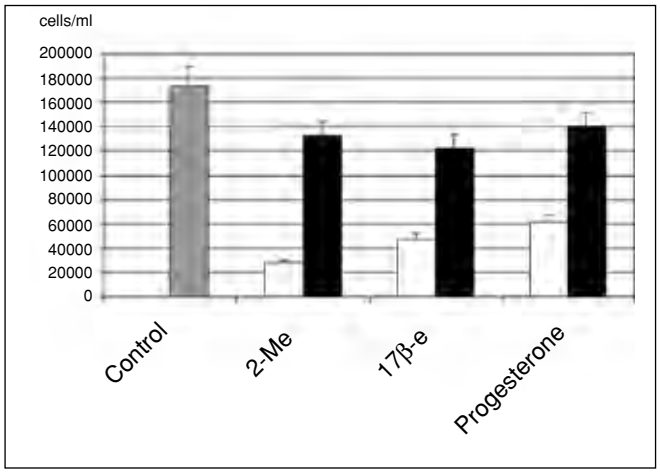


Figure 2. Reversibility assay on HUVECs for the effects of 2-methoxyestradiols (2-Me) 17β-estradiol (17-E) and progesterone. The light dotted bar represents the untreated control. Black bars represent steroid hormone treated probes after another 3 day incubation without hormones. White bars represent continuously hormone treated probes.

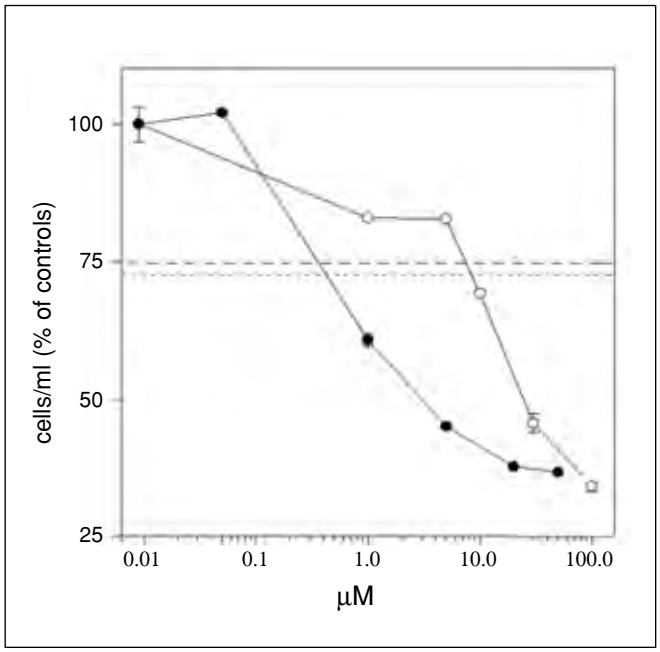


Figure 3. Effects of increasing concentrations of 2-methoxyestradiol (•), and 17β-estradiol (o), on the proliferation of HUVECs under confluent conditions. The number of unstimulated cells which are used as control cells are indicated.
2-methoxyestradiol : .....
17β-estradiol : ----------

lites on the bFGF-induced proliferation of HUVECs, HUAECs, HDMVECs, we determined the IC_{50} concentration of gonadal steroids 17-E, 2-Me and progesterone in these cells. As shown in Figure 1 and summarized in Table 1 these substances had a significant inhibiting effect on the proliferation of endothelial cells in this assay. Our results demonstrate that the strongest inhibition of endothelial cell proliferation was induced by 2-Me (HUVEC, IC_{50}: 1.2 μM) followed by progesterone (HUVEC, IC_{50}: 3.0) and 17-E (HUVEC, IC_{50}: 6.0). HUAECs and HDMVECs show also the strongest inhibition after incubation with 2-Me.

To determine whether the inhibitory effects of estradiol and its metabolites on endothelial cells were reversible, we incubated HUVECs for 4 days with 2 μM of 17-E, 1 μM 2-Me, and 2 μM progesterone, respectively. The number of viable cells was compared to the number of untreated cells after incubation without steroids in a Coulter counter. We could show that the inhibitory effects of all substances under investigation were reversible and the number of viable cells in the investigated probes was comparable to the untreated controls (Fig. 2).

Fotsis et al. described that 2-Me had an anti-angiogenic effect on bovine brain capillary endothelial cells (BBCE) and human endothelial cells only under growing/proliferating conditions but not under confluent conditions [19]. We therefore investigated the effects of steroids on human endothelial cells both under growing/proliferating and confluent conditions. We could show that under growing as well as under confluent conditions incubation of HUVECS with 17-E and 2-Me leads to an inhibition of cell growth (Fig. 3). The half maximal inhibitory concentration for 17-E and 2-Me were 25 μM and 3 μM, respectively. For 17-E the value is higher than for 17-E under proliferating conditions. However, the value for 2-Me is only slightly higher than under proliferating conditions suggesting that the action of 2-Me is widely independent of the proliferating status of the cells.

Table 1. Effects of various steroid hormones and metabolites on the proliferation of endothelial cells

Cells	IC_{50} [μM]		
	17β-estradiol	2-methoxyestradiol	progesterone
HUVEC	6.0	1.2	3.0
HUAEC	2.9	0.35	0.8
HDMVE	7.6	0.8	11.0

After demonstrating the growth inhibitory effects of 2-Me and 17-E, we wanted to determine if the observed effects were due to cytotoxicity or due to apoptotic mechanisms. First, we determined the number of vital cells after incubation for 24 h with 17-E, and 2-Me by a tetrazolium reduction assay. As shown in figure 4, the number of vital cells were not considerably reduced after incubation for 24 hours with 2-Me (10 μM), and only slightly and not significant with 17-E (10 μM). These results indicate that the observed growth inhibiting effects in endothelial cells are not due to the cytotoxicity of 2-Me or 17-E.

To further investigate 2-Me induced apoptosis, we analyzed the externalization of phosphatidylserine as an early event in the apoptotic pathway with the ApoAlert V-FITC assay by FACS analysis (19).

In the negative control without 2-Me incubation only 17.6% of the cells underwent apoptosis (Fig 5a). Using 10

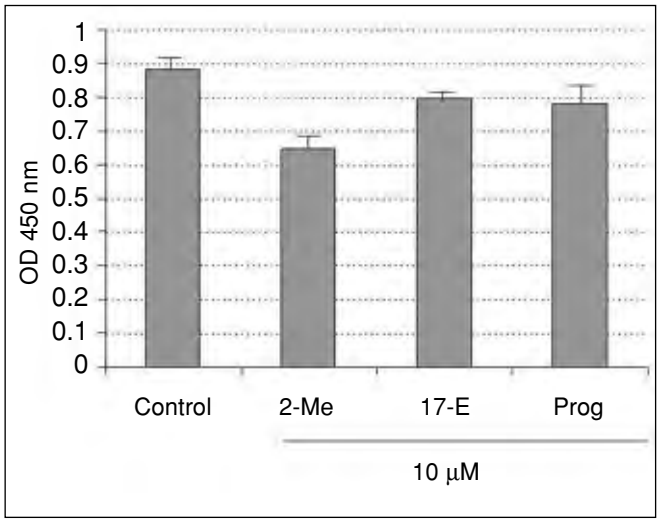


Figure 4. Cytotoxicity assay of gonadal steroids in HUVECs. Measurement of the reduction of uncoloured tetrazolium salts into colored formazan derivates at an optical density of 450 nm. Only living cells are capable of reducing compounds in the mitochondria, yielding a colored, soluble formazan dye. 2-Me: 2-Methoxyestradiol, 17-E: 17β-estradiol.

µM Actinomycin D as a positive control, 31.9% of the cells were apoptotic as shown in Figure 5b. After administration of 1 µM 2-Me into the medium for 24 hours, we observed that 22.08% of endothelial cells are apoptotic (Fig. 6a). After treatment of the cells with 5 µM and 10 µM 2-Me 24.9% and 23.02 % of the cells are apoptotic (Fig. 6b and Fig. 6c). This is an increase of 42.1% (at 5 µM) compared to the amount of apoptotic cells in the control experiment. These results demonstrates a significantly higher number of apoptotic cells in the 2-Me treated cultures than in the untreated controls pointing to apoptosis as the cause for cell death after 2-Me application. However, the number of apoptotic cells after administration of 1 µM 2-Me to the cultures is not as high as with cells which are treated with the apoptosis inducing agent actinomycin D (Fig. 5b) suggesting that higher doses of 2-Me or longer exposition might be more efficient.

Discussion

Several studies suggest a direct or indirect correlation between vessel formation and the steroids progesterone, and its metabolite 2-Me (20,25-30). 2-Me is a catecholestradiol endogenously produced in the human body, primarily generated by the peripheral conversion of estradiol. 2-Me circulates in the blood at a range of 30 pM in males to 30 nM in pregnant females, levels much lower than those at which antiproliferative and antiangiogenic effects occur (31). However, serum levels do not necessarily reflect the intracellular level of 2-Me, which remains unknown and which may be higher due to intracellular conversion of estradiol.

In the present study we show that progesterone, estradiols and their metabolites have strong inhibiting effects on endothelial cell proliferation at high concentrations. For the

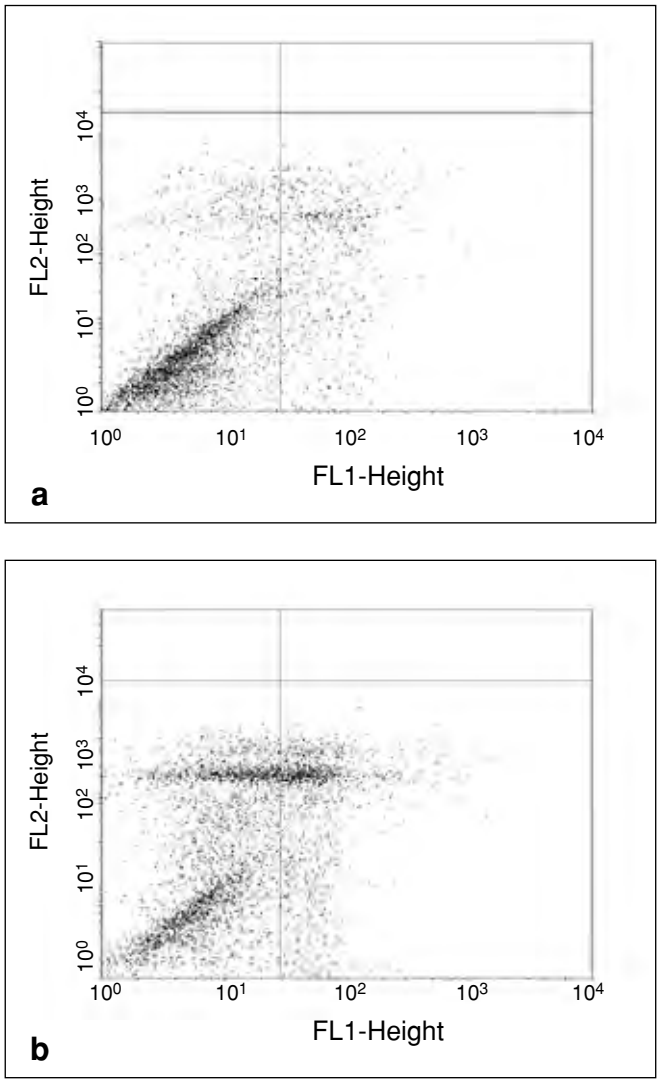


Figure 5. Flow cytometry density plots of representative mAb reactivities against HUVECs after 24 hours incubation without steroids (a) as a negative control and with Actinomycin D as a positive control (b). In the untreated control 17.6% of the cells were apoptotic, in the Actinomycin D treated control 31.9% of the cells were apoptotic.

substances under investigation, we found that 2-Me maximally inhibited the bFGF induced proliferation of HUVECs, HUAECs and HDMVECs.

These results strengthen the results of Fotsis and coworkers who demonstrated that 2-Me could inhibit proliferation of endothelial cells in vitro [14,18,19]. However, in contrast to the findings of Fotsis and coworkers we observed that this effect is independent of the proliferation status in HUVECs. Confluent as well as bFGF induced proliferating HUVECs were inhibited by 2-Me and 17-E as shown in Figures 3 and 5. However, the concentrations for a halfmaximal inhibition by 17-E and 2-Me were 4 fold (6 µM versus 25 µM) and 2 fold (1.2 µM versus 3 µM) higher in the confluent cultures, respectively. The differences between our data and those presented by Fotsis and coworkers might be explained in part by the different types of endothelial cells used (bovine brain capillary endothelial cells versus human umbilical vein endothelial cells).

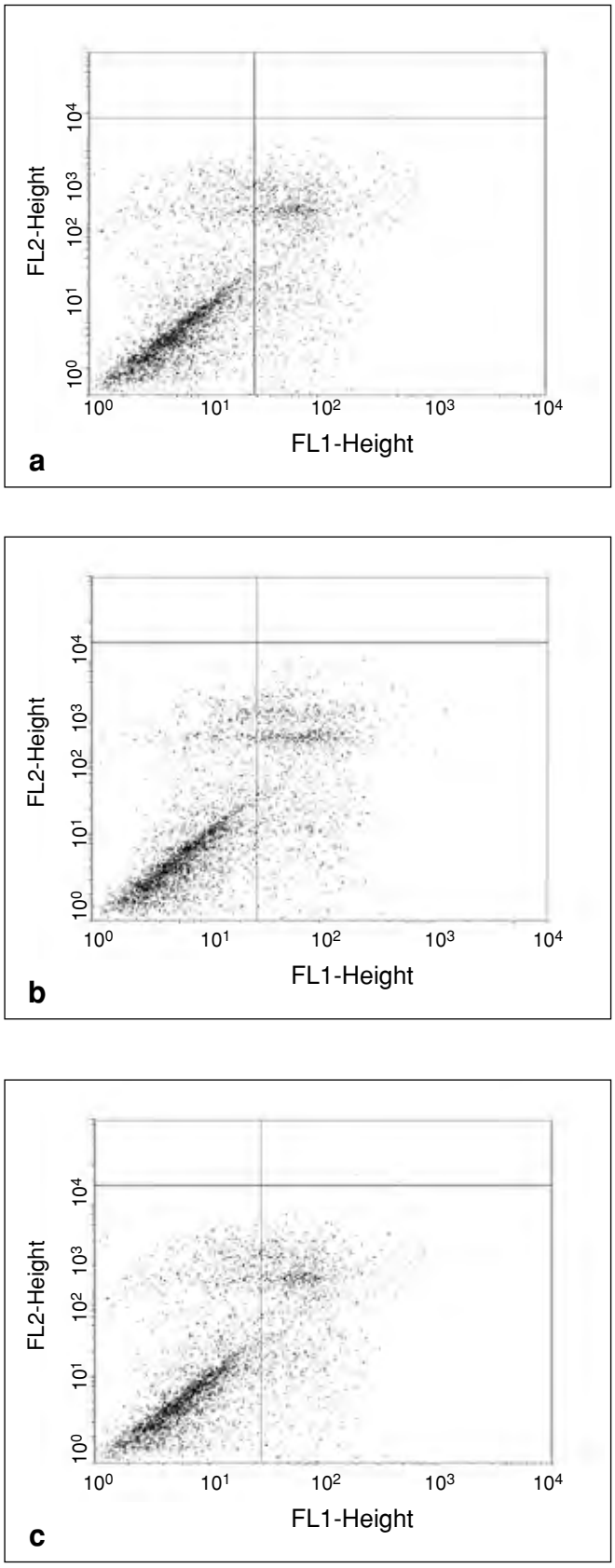


Figure 6. Flow cytometry density plots of representative mAb reactivities against HUVECs after 24 hours incubation with 1 µM 2-Me resulted in 22.08% apoptotic cells (a). Incubation with 5 µM 2-Me resulted in 24.9% apoptotic cells (b). Incubation with 10 µM 2-Me resulted in 23.02% apoptotic cells (c).

2-Me has repeatedly been reported to be a strong anti-angiogenic compound. This effect might be due to cytotoxic and/or apoptotic properties on endothelial cells [8,10]. In an attempt to find out if the observed growth inhibiting properties of progesterone, 17-E and 2-Me are based on cytotoxic or apoptotic mechanisms we undertook investigation with the RZ4U kit for cytotoxicity and with the APOALERT kit for detection of apoptosis. In the cytotoxicity assay we did not find any significant differences in the numbers of 17-E, progesterone or 2-Me treated cells compared to an untreated control. This indicates that these substances were not cytotoxic under the chosen conditions. In contrast the apoptosis assay revealed that a significant number of endothelial cells underwent apoptosis. Therefore these experiments show that the inhibition of cell proliferation by the steroids investigated is not due to cytotoxicity but rather appears to be the result of apoptotic mechanisms.

The process of apoptosis as the mechanism of cell death after 2-Me treatment has been observed in different cell types including various tumor cells. Different mechanism may lead to apoptotic cell death. A p53 mediated pathway was observed in lung cancer cells and in lymphoblast [9,23] and a p53 independent apoptotic pathway may be activated in gastric cancer cells mutated for the p53 gene [24].

It has not been clarified whether the induction of apoptosis by 2-Me is mediated via the estradiol receptor or if other unknown signaling mechanisms might be involved. Cushman and coworkers tested the affinity of estradiols and its metabolites to the estradiol receptor and find that the affinity of 2-Me to the estradiol receptor is extremely low compared to the affinity of estradiol [25]. Additionally, 2-Me similar inhibited both estradiol receptor positive and estradiol receptor negative cells [9]. It is still a matter of debate whether 2-Me transmits its signal via a receptor mediated pathway or if other non-genomic effects might contribute to the described inhibitory effect on cell proliferation. With respect to the apoptotic switch in programmed cell death the finding that the growth inhibiting properties of 2-Me is fully reversible, is somewhat conflicting but recent data by Levy and coworkers and Geske and coworkers [26,27] clearly point to the reversibility of apoptosis at least in the early stages when phosphatidylserine externalization occurs.

In conclusion, our results demonstrate the growth inhibiting effect of 2-Me in human endothelial cells by an apoptotic mechanism. The potency of 2-Me to inhibit the proliferation of angiogenic cells in vitro is emphasized and it is demonstrates that the antiproliferative effects of 2-Me are independent of the proliferation status of endothelial cells. Further studies are necessary to fully understand the physiologically and the potentially therapeutic role of 2-Me in cell proliferation.

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