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Estrogen receptor β mediates the inhibitory effect of estradiol on vascular smooth muscle cell proliferation

Tokumitsu Watanabe[a], Masahiro Akishita[b], Takashi Nakaoka[c], Koichi Kozaki[a], Yukiko Miyahara[a], Hong He[a], Yumiko Ohike[a], Teruhiko Ogita[d], Satoshi Inoue[a], Masami Muramatsu[e], Naohide Yamashita[c], Yasuyoshi Ouchi[a,*]

[a]*Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*
[b]*Department of Geriatric Medicine, Kyorin University School of Medicine, Tokyo 181-8611, Japan*
[c]*Department of Advanced Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan*
[d]*Department of Cardiovascular Medicine, University of Tokyo, Tokyo 113-8655, Japan*
[e]*Research Center for Genomic Medicine, Saitama Medical School, Saitama 350-1241, Japan*

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Abstract

Objectives: It has been demonstrated that 17β-estradiol (E2) has an inhibitory effect on the proliferation of vascular smooth muscle cells (VSMCs) through an estrogen receptor (ER)-dependent pathway. Both ER subtypes, classical ER (ERα) and the newly identified ER subtype (ERβ), are expressed in VSMCs. However, it remains unknown which receptor plays the critical role in the inhibitory effect on VSMC proliferation. **Methods and results:** We constructed replication-deficient adenoviruses bearing the coding region of human ERα, ERβ, and the dominant-negative form of ERβ (designated AxCAERα, AxCAERβ, and AxCADNERβ, respectively). Prior to infection with the adenoviruses, 100 nmol/l E2 attenuated DNA synthesis by up to 14% and transactivated the estrogen-induced expression of the desired mRNA in rat VSMCs. This was accompanied by increased transcriptional activity of estrogen responsive element in response to E2, and the increase was comparable between AxCAERα and AxCAERβ. When VSMCs were infected with AxCAERβ at a multiplicity of infection of 5 or higher, DNA synthesis as well as cell number decreased by 50% in response to E2, and the effect was abolished by co-infection with AxCADNERβ. In contrast, when VSMCs were infected with AxCAERα, the reduction in DNA synthesis was minimal. **Conclusions:** Our results indicate that ERβ is more potent than ERα in the inhibitory effect on VSMC proliferation.

Keywords: Atherosclerosis; Gene expression; Hormones; Receptors; Smooth muscle

1. Introduction

The proliferation of vascular smooth muscle cells (VSMCs) is a common feature associated with vascular proliferative disorders such as atherosclerosis and restenosis after balloon angioplasty [1]. Inhibition of VSMC growth is thus one therapeutic target for the prevention of vascular diseases. Estrogen exhibits a variety of actions on the vascular wall that could be involved in its atheroprotective effects [2,3]. These include the stimulation of nitric oxide production by endothelial cells [4] and the inhibition of VSMC proliferation [5–8]. However, results from recent randomized double-blind trials, which were conducted to evaluate the effect of hormone replacement therapy (HRT) in primary prevention [9] and in secondary prevention [10], have failed to show a protective effect of HRT on cardiovascular disease. These conflicting data might result from the prothrombotic effects of estrogen [11], which could abolish the beneficial effects of estrogen on vascular function. Additionally, progestin, combined with estrogen to decrease the risk of endometrial cancer during HRT, might exert prothrombotic and proinflammat-

*Corresponding author. Tel.: +81-3-5800-8830; fax: +81-3-5800-6530.
E-mail address: youchi-tky@umin.ac.jp (Y. Ouchi).

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ory effects. So far, the protective effects of estrogen alone on cardiovascular diseases remain unknown.

Most of the effects of estrogen are thought to be mediated by the estrogen receptor (ER), a member of the intra-nuclear receptor family. A new subtype of ER, ERβ, was discovered in 1996 [12], and has a somewhat different expression and localization patterns and transcriptional activity in reproductive and non-reproductive organs from those of classical ERα [13]. The ER subtypes may provide a clue to answering the question of why estrogen exerts differential effects in various cells and tissues; that is, estrogen stimulates proliferation in MCF-7 breast cancer cells [14] and osteoblastic cells [15], but inhibits proliferation in VSMCs. Morey et al. showed that, in VSMCs, the growth inhibitory effect of estrogen can be blocked by the nonspecific estrogen receptor antagonists tamoxifen [6] and ICI 182,780 [8]. However, it remains unknown which ER subtype mediates the growth inhibitory effect of estrogen in VSMCs, where both ER subtypes are expressed [16–18].

Also, in vivo studies using genetically engineered mice have provided insufficient information on this issue. Estrogen inhibites VSMC proliferation of the medial area in response to vascular injury in ERα knockout mice [19] as well as in ERβ knockout [20] and double knockout mice [21]. In contrast, estrogen has no detectable effect on VSMC proliferation in fully null ERα knockout mice [22], suggesting that a splice variant of the ERα gene in the previous knockout mice lines plays a role. However, some points remain unclear in the study. Would the function of a splice variant, scarcely expressed in the vascular wall, really be as efficient as that of wild-type ERα? VSMC proliferation is inhibited in newly generated ERα knockout mice in an estrogen-independent manner as compared to wild-type mice [22]. This result suggests that ERα could exert ligand-independent VSMC proliferation, an interesting, but not established, concept.

In the rat carotid injury model, ERβ is predominantly expressed after injury [23], and the isoflavone phytoestrogen genistein, which showed a 20-fold higher binding affinity to ERβ than to ERα, exhibited a vasculoprotective effect. Taken together, ERβ might be a main mediator for the estrogen-mediated vasculoprotective effect. In the present study, to clarify which ER subtype plays the pivotal role in the inhibitory effect of estrogen on VSMC proliferation, we used adenovirus vectors to transfer ER subtypes into VSMCs. As reported previously, estradiol (E2) attenuates DNA synthesis dose-dependently. Adenovirus-mediated overexpression of ERβ in VSMCs augments growth inhibition in a ligand-dependent manner.

2. Methods

2.1. Cell culture

Rat VSMCs were harvested from the aortae of 8-week-old Wistar male rats by enzymatic dissociation according to the modified method of Chamley et al. [24]. All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo. Human aortic VSMCs were purchased from Clonetics (Cat. #CC-2571). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Laboratory, Tokyo, Japan) supplemented with 10% fetal bovine serum (Intergen Co., Purchase, NY, USA), 25 mM HEPES (pH 7.4), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Rat VSMCs at six to 10 passages were used in the experiments. At the time of the experiments, we used dextran-coated charcoal-stripped FBS (DCC-FBS) and phenol red-free RPMI1640 medium for rat VSMCs and M199 medium for human VSMCs to avoid contamination with steroids and estrogen receptor agonist. All dishes used in this study were purchased from Asahi Techno Glass Co., Ltd., Tokyo.

2.2. Construction of adenovirus vector carrying estrogen receptor subtypes and transfer into VSMCs

Replication-deficient adenovirus vectors carrying the CMV-IE enhancer, chicken β-actin promoter, and the coding region of human ERα, ERβ, or the dominant-negative form of ERβ [25] were constructed by use of an adenovirus expression vector kit (Takara Shuzo Co., Kyoto, Japan) as described before [26], and are denoted AxCAERα, AxCAERβ, and AxCADNERβ, respectively. VSMCs were exposed to different multiplicities of infection (MOI) of either AxCAERα, AxCAERβ, AxCADNERβ, or a replication-deficient recombinant adenovirus carrying the *Escherichia coli* β-galactosidase gene (AxCALacZ) for 2 h in DMEM with 5% FBS. The cells were then rinsed with phosphate-buffered saline once, and used for the experiments.

2.3. RNA isolation, reverse transcription polymerase chain reaction (RT-PCR), and Northern blot analysis

For RT-PCR, total RNA was prepared from VSMCs and, as a positive control, rat ovary, using Isogen (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Then, 1 μg total RNA was reverse transcribed into cDNA, and 1/20 of the product was amplified for 35 cycles. Negative control RT-PCR reactions were performed by omitting reverse transcriptase. The primer pairs used in PCR were: CTAAGAAGAATAGCCCCGCC (forward, +1126 to +1145) and CAGACCAGACCAATCATCAGG (reverse, +1402 to +1382) for rat ERα (GenBank, accession number NM_012689), and CGACTGAGCACAAGCCCAAATG (forward, +76 to +97) and ACGCCGTAATGATACCCAGATG (reverse, +353 to +332) for rat ERβ (GenBank, accession number AB012721). Both PCR products were subsequently sequenced, and were used as the probes for rat ERα and ERβ.

For Northern blotting, VSMCs were plated on 10 cm diameter dishes, and infected with adenovirus bearing either ER subtype at 70–90% confluence. At 24 h after infection, VSMCs were harvested using ISOGEN. The RNA was fractionated on 1.3% formaldehyde-agarose gel and transferred to nylon filters (Hybond-N; Amersham Life Science Inc.). The filters were hybridized at 68 °C for 2 h with a random-primed ^{32}P-labeled human ER cDNA probe in QuikHyb solution (Stratagene) and autoradiographed. The products digested by EcoRI and PVUII from human ERα plasmid and EcoRI from human ERβ plasmid were used as the human ERα and human ERβ probe, respectively.

2.4. Western blot analysis

Cells were washed quickly with phosphate-buffered saline twice, and lysed in RIPA buffer: 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors cocktail (Complete, Mini; Boehringer Mannheim). The samples were separated on 12% SDS–PAGE, electroblotted onto nitrocellulose membranes, and immunoblotted with anti-ERα polyclonal antibody (H-184; Santa Cruz, 1:1000 dilution), anti-ERβ monoclonal antibody, CWK-F12 (kindly provided by Dr. Benita S. Katzenellenbogen, Department of Molecular and Integrative Physiology, University of Illinois College of Medicine, 1:1000 dilution), or cyclin A polyclonal antibody (C-19; Santa Cruz, 1:1000 dilution). Antibody was detected with a horseradish peroxidase-linked secondary antibody using an enhanced chemiluminescence system (Amersham Life Science Inc.).

2.5. Transfection and luciferase assays

We used the ERE-TK-Luc reporter plasmid and a firefly luciferase reporter vector as previously described [24]. VSMCs were transfected with ERE-TK-Luc reporter plasmid and pRL-SV40 control plasmid using FuGENE6 (Roche) for 24 h according to the manufacturer's instructions. Then, VSMCs were incubated in phenol-red-free RPMI1640 containing 0.1% DCC-FBS for 24 h, and exposed to 1–100 nmol/l E2 (water-soluble 17β-estradiol; Sigma–Aldrich Japan), 10–1000 nmol/l ICI 182,780 or vehicle, β-cyclodextrin solution (Sigma) as a vehicle for water-soluble E2, for an additional 24 h. We measured two kinds of luciferase activity using a dual-luciferase reporter assay system (Promega) according to the manufacture's protocol, and the ratio of firefly luciferase activity to that of Renilla luciferase in each sample was used as a measure of normalized luciferase activity. Each experiment was repeated at least three times.

2.6. Measurement of [^{3}H]thymidine incorporation into DNA of VSMCs

VSMCs seeded onto 24-well tissue culture plates were grown until 70–90% confluence, and then made quiescent by culturing them in phenol-red-free RPMI1640 medium (Gibco) for 24 h. Then, the cells were stimulated with 5% DCC-FBS in the presence of E2 (water-soluble 17β-estradiol; Sigma–Aldrich Japan) or vehicle for 24 h, followed by pulse-labeling with 1 μCi/ml [^{3}H]thymidine for 3 h. [^{3}H]Thymidine incorporated into DNA was determined as previously described [5].

2.7. Number of VSMCs

VSMCs were seeded onto six-well multiplates and cultured until a confluent state was obtained. After infection of VSMCs with adenovirus vectors, the medium was replaced with phenol-red-free RPMI1640 to arrest the growth. After 24 h, the medium was replaced again with phenol-red-free RPMI1640 containing 5% DCC-FBS with E2 or vehicle. After incubation for 48 h, the cells were trypsinized and suspended. Then the number of cells was determined using a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL, USA).

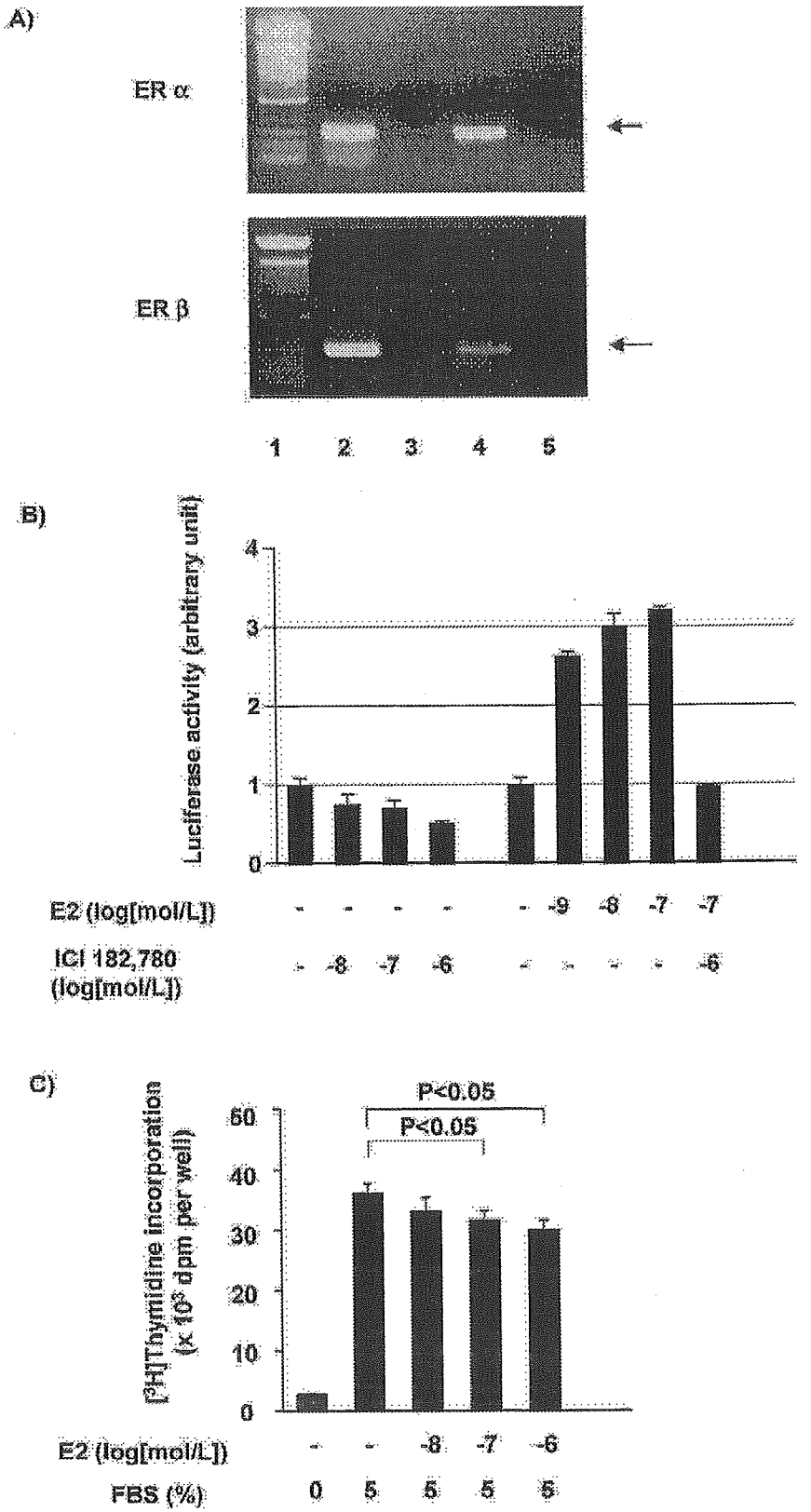
2.8. Statistical analysis

The dose–response effect of E2 or adenoviruses on DNA synthesis in VSMCs and the luciferase activity in E2-treated VSMCs were analyzed using one-way ANOVA. If a statistically significant effect was found, Newman–Keuls' test was performed to isolate the difference between groups. A value of $P<0.05$ was considered statistically significant. All data in the text and figures are expressed as mean±S.E.

3. Results

3.1. Endogenous expression of ER subtypes in VSMCs and effect of E2 on VSMC growth

To investigate the endogenous expression of ER in rat VSMCs, RT-PCR amplification was performed. Both rat ERα and ERβ were expressed in VSMCs (Fig. 1A). Next, we examined the transcriptional activity of endogenous ER by means of the luciferase activity of the ERE reporter plasmid, and the inhibitory effect of E2 on VSMC proliferation by evaluating DNA synthesis. E2 at 1–100 nmol/l augmented the luciferase activity of ERE by approximately three-fold compared to vehicle, and this increase was abolished by the nonselective pure ER antagonist ICI 182,780 (AstraZeneca) (Fig. 1B). At these concentrations, E2 inhibited the proliferation of VSMCs dose-dependently (Fig. 1C). In the absence of E2, ICI 182,780 inhibited the luciferase activity dose-dependently by up to 50% compared to vehicle (Fig. 1B), but did not influence thymidine incorporation into VSMCs at concentrations of 10–1000 nmol/l (data not shown). This result indicates that there may be some leakage of estrogenic activity from cell


A)
ER α
ER β
1 2 3 4 5
B)
Luciferase activity (arbitrary unit)
0 1 2 3 4
E2 (log[mol/L]) - - - - - -9 -8 -7 -7
ICI 182,780 (log[mol/L]) - -8 -7 -6 - - - - -6
C)
[3H]Thymidine incorporation (x 10³ dpm per well)
0 10 20 30 40 50 60
P<0.05
P<0.05
E2 (log[mol/L]) - - -8 -7 -6
FBS (%) 0 5 5 5 5

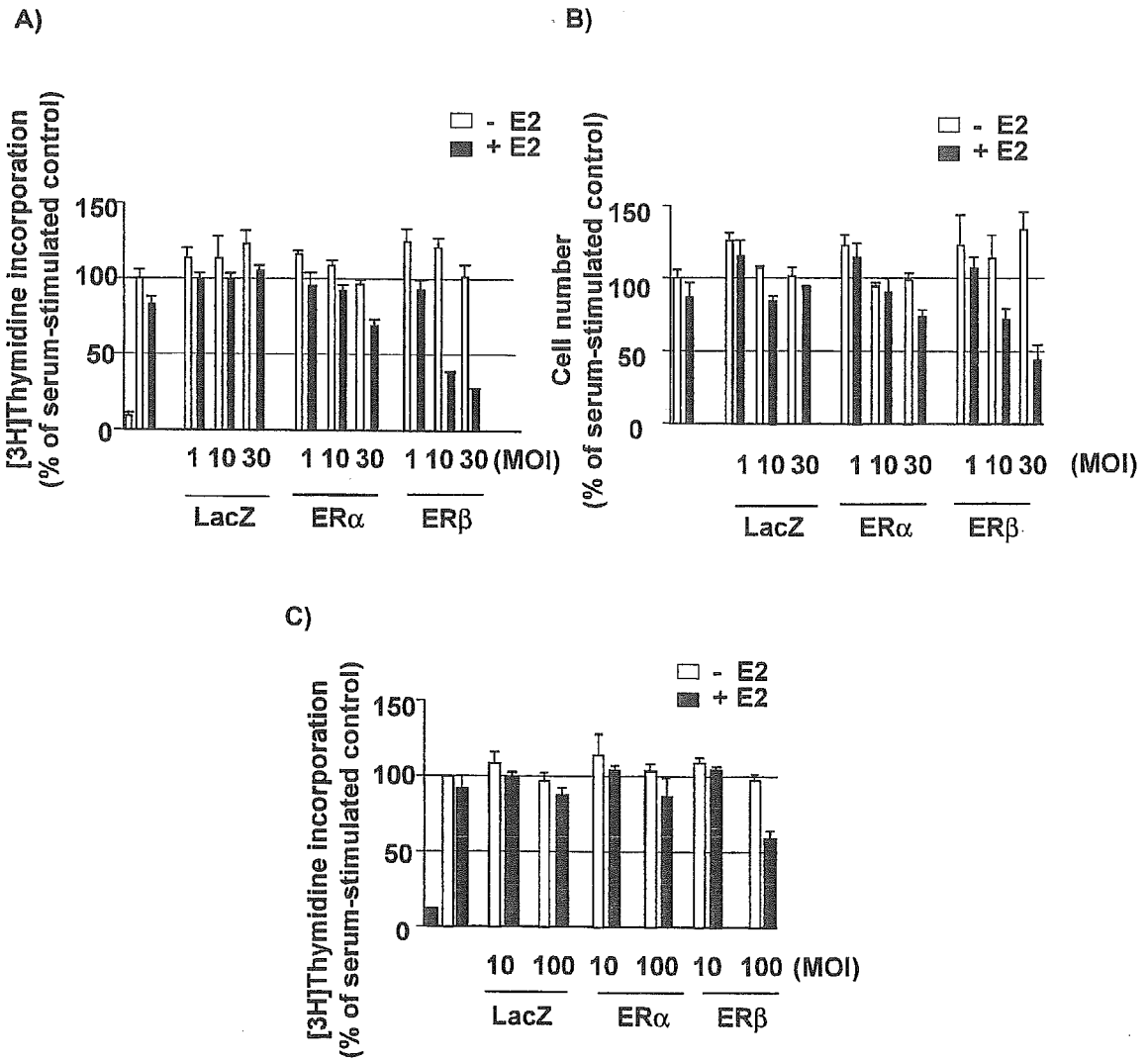


Fig. 2. Inhibition of VSMC growth by adenovirus-mediated transfer of ER gene. (A) Rat VSMCs seeded onto a 24-well plate were exposed to DMEM containing either AxCALacZ, AxCAERα, or AxCAERβ (1, 10, and 30 MOI, increasing left to right) for 2 h, and were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined after 24 h of stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/l E2, respectively. The three left-sided bars indicate non-infected VSMCs serum free, 5% DCC-FBS in the absence of E2, and 5% DCC-FBS in the presence of 100 nmol/l E2, respectively. Results are shown as mean±S.E. (n=4). Similar results were obtained in three independent experiments. (B) VSMCs seeded onto a six-well plate were exposed to DMEM containing either AxCALacZ, AxCAERα, AxCAERβ, or AxCAERDNβ (1, 10 and 30 MOI, increasing left to right) for 2 h, and were serum-deprived for 24 h. Cell numbers were counted after 48 h of stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/l E2. The two left-sided bars indicate non-infected VSMCs with 5% DCC-FBS in the absence of E2 and with 5% DCC-FBS in the presence of 100 nmol/l E2, respectively. Results are shown as mean±S.E. (n=3). Similar results were obtained in three independent experiments. (C) Human aortic VSMCs seeded onto a 24-well plate were exposed to DMEM containing either AxCALacZ, AxCAERα, or AxCAERβ (10, and 100 MOI, increasing left to right) for 2 h, and were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined after 24 h of stimulation with 20% DCC-FBS in the presence or absence of 100 nmol/l E2, respectively. The three left-sided bars indicate non-infected VSMCs serum free, 20% DCC-FBS in the absence of E2, and 20% DCC-FBS in the presence of 100 nmol/l E2, respectively. Results are shown as mean±S.E. (n=3). *P<0.05 vs. E2 (−). Similar results were obtained in three independent experiments.

Fig. 1. The endogenous expression of ER subtypes, transcriptional activity of ER, and inhibitory effect of E2 on DNA synthesis in rat VSMCs. (A) RT-PCR was performed using the cDNA of rat ovary as a positive control with (lane 2) or without reverse transcriptase (lane 3) and that of rat VSMCs with (lane 4) or without reverse transcriptase (lanes 5). A single band of predicted size (277 bp for ERα and 278 bp for ERβ, indicated by an arrow) was detected in lane 2 and lane 4. Lane 1 shows the molecular weight marker. (B) VSMCs were transfected with luciferase reporter plasmid containing ERE and pRL-SV40 control plasmid. Twenty-four hours after transfection, the cells were treated with 1–100 nmol/l E2 and/or 10–1000 nmol/l ICI182,780 for 24 h. The values were normalized to the vehicle treatment. Results are shown as mean±S.E. (n=3). *P<0.01 vs. E2 (−). (C) Serum-starved VSMCs were stimulated with 5% DCC-FBS in the absence or presence of 10–1000 nmol/l 17β-estradiol for 24 h. [^{3}H]Thymidine incorporation into DNA was determined by pulse-labeling for the last 3 h of incubation. Results are shown as mean±S.E. (n=6).

culture dishes [27,28] detected in the luciferase assays, but the activity is not strong enough to influence VSMC proliferation.

3.2. Effect of adenovirus-mediated transfer of the ER subtype gene on growth of VSMCs

To examine the effect of ERα and ERβ gene transfer into VSMCs, we constructed a replication-deficient adenovirus carrying the ER gene, AxCAERα, AxCAERβ, or AxCADNERβ. When AxCALacZ was introduced into VSMCs at more than 30 MOI, DNA synthesis was reduced in a MOI-dependent manner (data not shown). Therefore, we examined DNA synthesis at 30 MOI or less, at which the adenovirus itself did not affect DNA synthesis. When AxCAERβ was introduced into VSMCs, DNA synthesis did not change in the absence of E2. However, in the presence of 100 nmol/l E2, DNA synthesis of VSMCs infected with AxCAERβ decreased strongly compared to that of VSMCs treated with vehicle, in a MOI-dependent manner (Fig. 2A). In contrast, VSMCs infected with AxCAERα at 10 MOI or less did not show an additional reduction in DNA synthesis in the presence of E2, although an inhibitory effect was seen in VSMCs infected with AxCAERα at 30 or higher MOI (Fig. 2A and data not shown). In parallel, the increase in VSMC number stimu-

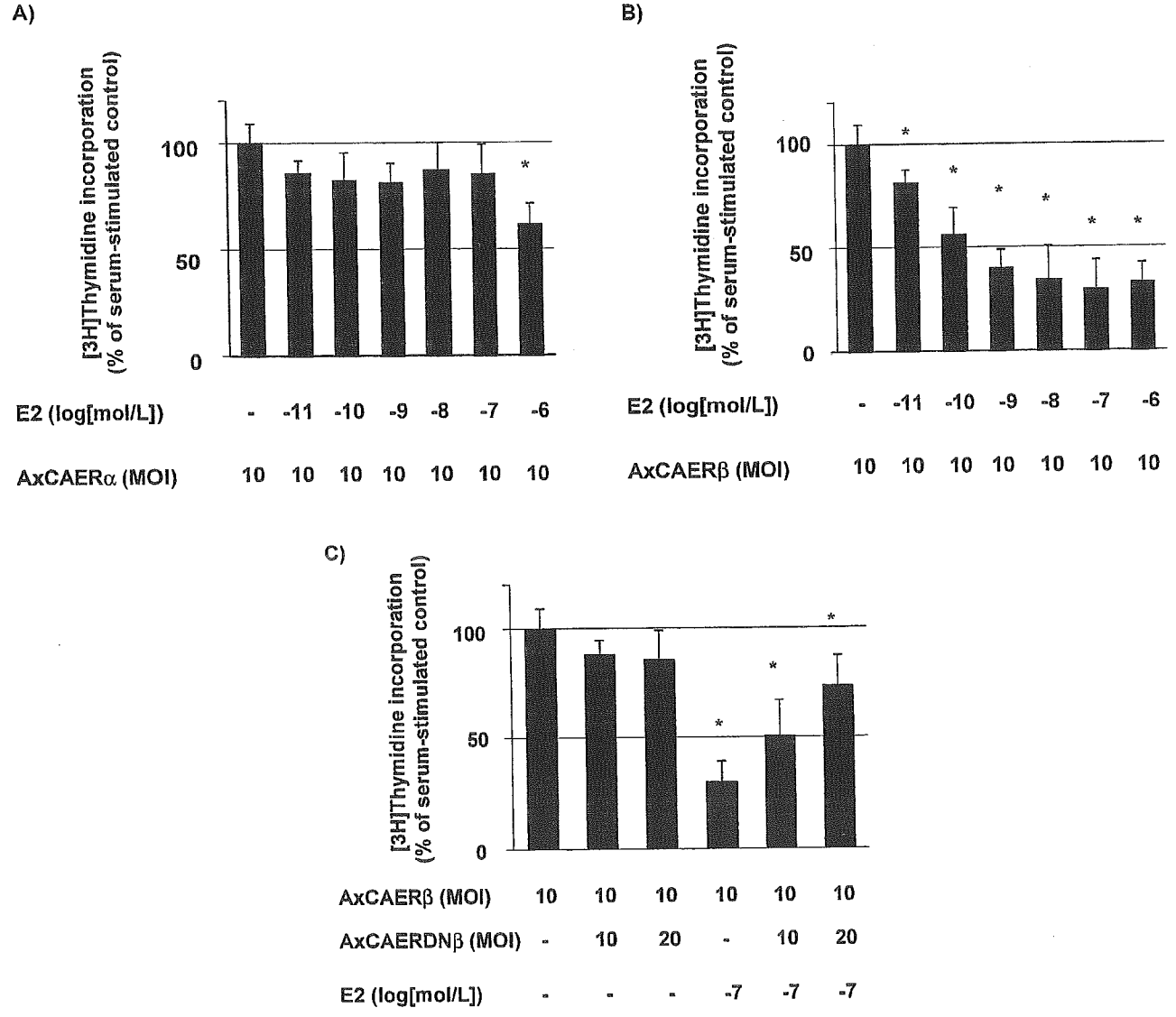


Fig. 3. Dose–response of E2 and receptor dependence of the inhibitory effect of DNA synthesis on adenovirus-mediated transfer of ER genes. VSMCs seeded onto a 24-well plate were exposed to DMEM containing 10 MOI of AxCAERα (A) or AxCAERβ (B) for 2 h, or 10 MOI of AxCAERβ and the indicated MOI of AxCADNERβ (C). After infection, VSMCs were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined 24 h after stimulation with 5% DCC-FBS in the absence or presence of the indicated concentrations of E2. Results are shown as mean±S.E. (n=4).

lated with 5% DCC-FBS for 48 h was attenuated in VSMCs infected with AxCAERβ at 10 and 30 MOI in the presence of E2, but it was not significant in VSMCs infected with AxCALacZ or AxCAERα (Fig. 2B). To exclude the possibility that the findings might be specific for rat VSMCs, we tested human aortic VSMCs and found that the results were comparable in human aortic VSMCs (Fig. 2C). Also, in VSMCs infected with AxCAERβ at 10 MOI, DNA synthesis was significantly inhibited by 0.01–1000 nmol/l E2 in a concentration-dependent manner (Fig. 3B). In contrast, this inhibitory effect was not observed in VSMCs infected with AxCAERα, except in the presence of 1 μmol/l E2 (Fig. 3A). To examine whether the inhibitory effect in VSMCs overexpressing ERβ is actually mediated through ERβ, AxCADNERβ was co-infected with AxCAERβ. The ~70% reduction in DNA synthesis that was observed when AxCAERβ alone was infected was attenuated by co-infection of AxCADNERβ MOI-dependently (Fig. 3C). We also examined the effect of ERα overexpression on ERβ-mediated inhibition of VSMCs. However, AxCAERα at up to 10 MOI did not influence the growth inhibition exerted by AxCAERβ at 10 MOI in the presence of 100 nmol/l E2 (data not shown).

3.3. Production of ER genes and transcriptional activity of ERE in VSMCs infected with ER genes

We examined the mRNA expression of human ERα, ERβ and DNERβ by Northern blot analysis (Fig. 4A and data not shown). Neither ERα nor ERβ mRNA was seen in non-infected VSMCs (data not shown), although both were detected by RT-PCR. Infection of VSMCs with AxCAERα or AxCAERβ induced mRNA expression abundantly in a MOI-dependent manner (Fig. 4A). Similar results were obtained when the membranes were hybridized with the probes for rat ERα and ERβ, indicating that the mRNA expression of endogenous ER was undetectable by Northern blot analysis. Production of the ERα and ERβ protein was confirmed by Western blot analysis (Fig. 4B). The bands corresponding to ERα (65 kD) or ERβ (55 kD) were seen in VSMCs infected with AxCAERα, or VSMCs infected with AxCAERβ, respectively (Fig. 4B) and also in MCF-7 cells which were used as a positive control (data not shown). In parallel with the mRNA expression, the protein expression of the ER subtype was undetectable in non-transfected VSMCs and was increased by overexpression MOI-dependently. We also checked the protein level of both ER subtypes in non-infected cells after the addition of E2. However, E2 did not affect the protein level of either ER subtype under our experimental conditions (data not shown).

To check whether overexpressed ER functions as a transcription factor in VSMCs, the transcriptional activity of ERE was examined (Fig. 5A). VSMCs infected with AxCAERα or AxCAERβ at 10 MOI showed a significant increase in transcriptional activity in the presence of E2 ($P<0.01$ vs. VSMCs infected with 10 MOI AxCALacZ), indicating that both subtypes can work as transcription factors. The results in COS-7 cells (Fig. 5B), in which no endogenous ER is expressed, are clear-cut and suggest that adenovirus infection can induce ERα and ERβ to a similar extent in terms of transcriptional activity. Compared with COS-7 cells, the additional activity caused by ER overexpression was small in VSMCs. The increase, however, was completely abolished by co-infection with AxCADNERβ, suggesting that the transcriptional activity both in non-infected and infected VSMCs in response to E2 was specific for ER.

3.4. Effect of E2 on cyclin A expression

The expression of cyclin A protein in VSMCs was

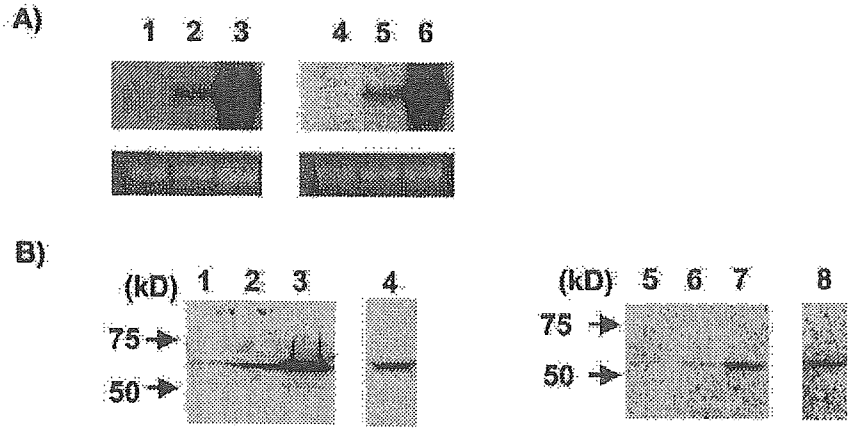


Fig. 4. Induction of ER mRNA and protein in VSMCs by adenovirus-mediated transfer of ER genes. (A) VSMCs were infected without (lanes 1 and 4), or with 10 and 100 MOI of AxCAERα (lanes 2 and 3, respectively) or AxCAERβ (lanes 5 and 6, respectively). Total RNA was extracted from VSMCs, and Northern blot analysis was performed with 15 μg total RNA per lane. The membrane was hybridized to ^{32}P-labeled ERα or β (upper lane). The 18S RNA is shown as the loading control (lower lane). (B) VSMCs were infected without (lanes 1 and 5) or with 10 and 100 MOI of AxCAERα (lanes 2 and 3, respectively) or AxCAERβ (lanes 6 and 7, respectively). Positive controls are shown in lane 4 (MCF-7 cells) and lane 8 (rat ovary). Western blot analysis was performed with 40 μg of protein per lane by using an anti-ERα polyclonal antibody or anti-ERβ monoclonal antibody.

examined 18 h after the addition of 100 nmol/l E2. The expression was increased by the addition of serum and was attenuated in VSMCs infected with AxCAERβ in the presence of E2. In contrast, E2 did not significantly inhibit cyclin A protein expression in VSMCs infected with AxCALacZ or AxCAERα (Fig. 6).

4. Discussion

Our previous work and several articles by other investigators have clearly demonstrated that E2 inhibits the proliferation of VSMCs [5–8]. Recently, it was reported that ER antagonists, ICI182,780 [8] and tamoxifen [6], antagonized the inhibitory effect of estrogen, indicating that the effect was mediated by ER. However, these inhibitors are non-selective for the ER subtype, and tamoxifen exerts a partial agonistic effect in some tissues [29]. Thus, it remained unclear which receptor is involved in the inhibitory effect of estrogen on VSMC proliferation.

There are several reports focusing on the effect of estrogen on cell proliferation using adenoviruses carrying ER into the breast cancer cell line or the pituitary lactrope cell line [30–32]. In MDA-MB 231 cells, an ER-negative human breast cancer cell line, overexpression of ERα inhibited proliferation hormone-dependently, whereas that of ERβ inhibited proliferation ligand-independently [31]. Also, overexpression of wild-type ERα in a pituitary lactrope cell line inhibited proliferation and induced apoptosis [32]. In contrast to these reports, overexpression of the dominant-negative form of ERα inhibited the proliferation of MCF-7 cells, in which endogenous ERα was expressed, and the proliferation was increased in an estrogen-dependent manner [30]. Although the question of ERα overexpression resulting in growth inhibition depending on the cell line is unresolved, the use of recombinant adenoviruses in this study enabled us to induce ERα and ERβ abundantly in VSMCs in which the endogenous expression of both ER subtypes was low.

The present study demonstrates direct evidence that ERβ is involved in the control of VSMC proliferation. The inhibitory effect of ERβ overexpression was restored by co-infection of dominant negative ERβ, indicating that this phenomenon actually resulted from signaling via ERβ. This dominant negative form of ERβ has an inhibitory effect on the transcriptional activity of both wild-type ERα and ERβ, as demonstrated by Ogawa et al., who originally made these constructs [25]. They made the C-terminal truncated ERβ, and showed that this dominant negative form of ERβ can bind to both wild-type ERα and ERβ. Accordingly, the dominant negative ERβ we used can inhibit not only the homodimerization of wild-type ER, but also heterodimerization of ERα and ERβ. The downstream effect was unknown, although competition for ERE binding, formation of inactive heterodimers with wild-type ER and specific transcriptional silencing could be assumed.

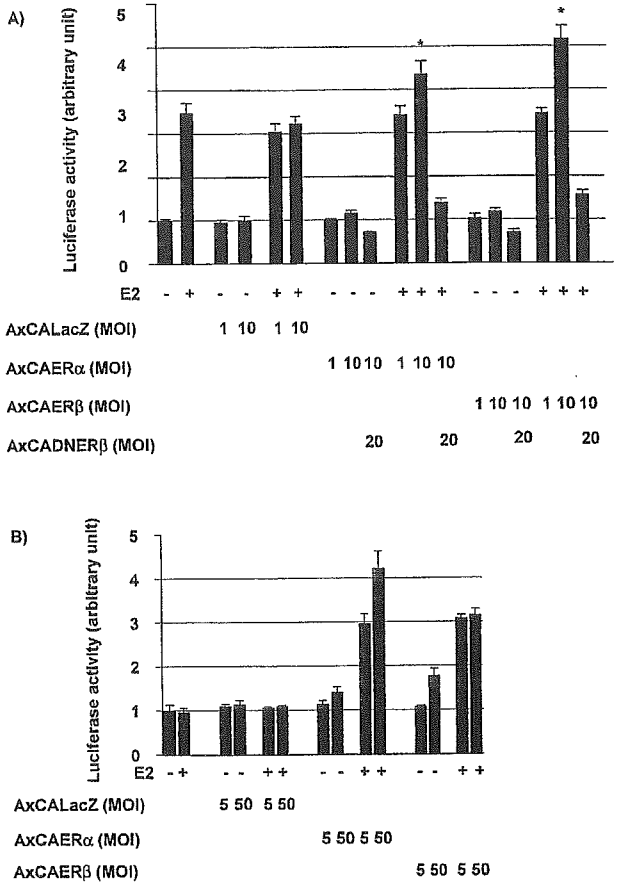


Fig. 5. Influence of overexpression of ERs on promoter activity of ER responsive enhancer elements in VSMCs and COS-7 cells. (A) VSMCs were infected with AxCALacZ, AxCAERα, AxCAERβ, or AxCADNERβ for 2 h, and were then transfected with luciferase reporter plasmids. Twenty-four hours after transfection, the cells were treated with or without 100 nmol/l E2 for 24 h. *$P<0.01$ vs. VSMCs infected with 10 MOI of AxCALacZ with E2. (B) COS-7 cells were infected with AxCALacZ, AxCAERα, or AxCAERβ for 2 h, and were then transfected with luciferase reporter plasmids. Twenty-four hours after transfection, the cells were treated with or without 100 nmol/l E2 for 24 h. The values were normalized to those in non-infected VSMCs without E2. Results are shown as mean±S.E. ($n=3$).

Surprisingly, the inhibitory effect of E2 was seen even at 10 pmol/l in VSMCs infected with ERβ. On the other hand, what is the role of ERα in VSMC proliferation? When ERα was infected into growth-arrested VSMCs, no proliferative response to E2 was seen (data not shown). Also, when both ERα and ERβ were co-infected into VSMCs, the inhibitory effect of ERβ was not affected. These results indicate that ERα does not show stimulatory effects or antagonize ERβ in terms of VSMC growth. When VSMCs were infected with AxCAERα at a higher MOI (30 or 100 MOI), an inhibitory effect appeared (Fig. 2A and data not shown). Taken together, ERα may have a weak inhibitory effect on VSMC proliferation.

The divergent effects of the ER subtypes may be

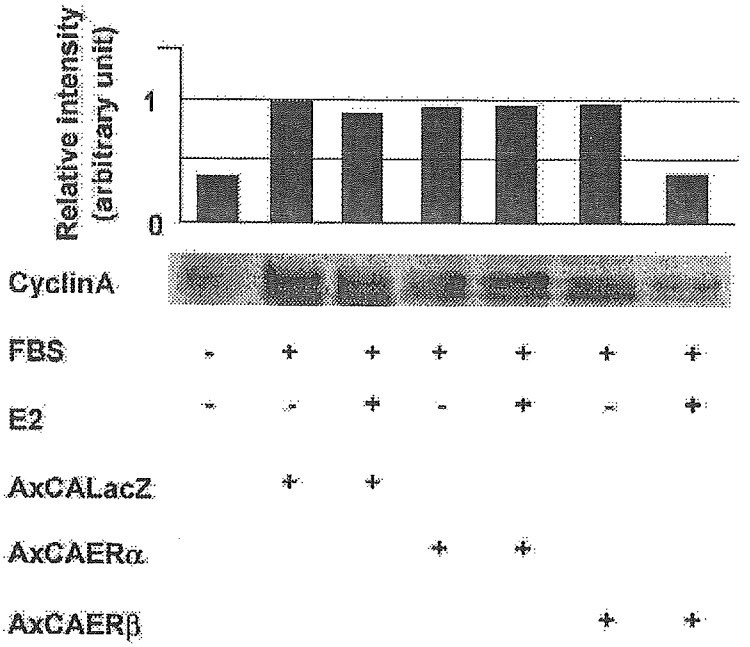


Fig. 6. Downregulation of cyclin A protein by E2 in VSMCs infected with AxCAERβ. VSMCs seeded onto 10 cm dishes were exposed to DMEM containing 10 MOI of AxCALacZ, AxCAERα, or AxCAERβ for 2 h. After infection, VSMCs were serum-deprived for 24 h. Samples were harvested 18 h after stimulation with 5% DCC-FBS in the absence or presence of 100 nmol/l E2. Western blot analysis was performed with 30 μg of protein per lane by using an anti-cyclin A polyclonal antibody. In the upper panel, levels of cyclin A protein expression in the membrane were measured by densitometry, and were plotted in comparison with that in serum-stimulated VSMCs without E2. Similar results were obtained in three independent experiments.

explained by the differential induction of estrogen response genes [33], or they may be due, in part, to the differential recruitment of transcriptional co-factors. A difference in ligand-binding affinity has also been reported [34]. Recently, it was also reported that ERβ, but not ERα, binds MAD2, a cell cycle spindle assembly checkpoint protein. The interaction is not altered by the absence or presence of E2, and thus ERβ is thought to function as a component of the spindle checkpoint assembly, not as a transcriptional factor [35]. With respect to cell growth, c-myc proto-oncogene expression was decreased in breast cancer cells infected with ERα, but was not changed in cells infected with ERβ, although the transcriptional activity is similar in cells infected with different ER subtypes [31]. In our study, there is also a discrepancy between the ERE luciferase activity and thymidine incorporation in terms of dose-dependency and the differential roles of ER subtypes. The reason for this is unknown, but similar findings have been reported [31]. We can put forward an hypothesis: some machinery, such as the co-factor for ER, would be limited in VSMCs, and the overexpression of ER could increase the ERE transcription activity to a small extent. By contrast, the signaling pathway mediating growth inhibition might manipulate some response gene that did not contain the typical ERE or non-genomic factors [6,36]. Thus we checked a cell cycle regulated gene, cyclin A. This molecule is important in the G1/S transition and in the S and G2/M phases of the cell cycle and plays a critical role in DNA replication [37]. Although the direct interaction between ERβ and cyclin A could not be clarified, cyclin A might be one of the specific response genes for ERβ in VSMCs. With respect to signaling pathways, it was reported that E2 had an inhibitory effect on VSMC proliferation via the inhibition of mitogen-activated protein kinase (MAPK) activity [6], an increase in the expression of MAPK phosphatase-1 and the activity of two Src homology 2 domain-containing cytosolic tyrosine phosphatases [38], or the cyclic AMP–adenosine pathway [7]. These signaling pathways are attributable, in part, to the non-genomic action of E2 [6,38]. We have tested whether the inhibition of MAPK activity could be involved in the inhibition of VSMC growth. However, under our study conditions, E2 did not affect ERK activity regardless of infection. Further investigations are required to clarify the specific signaling pathway by which ER subtypes exert differential effects on VSMC proliferation.

The in vivo relevance of our findings should be discussed. Studies on vascular injury using genetically engineered mice, such as ERα knockout [19], ERβ knockout [20], ERα and β double knockout mice [21] and fully null ERα knockout [22], are not yet conclusive in addressing the role of ER subtypes. In rats, ERβ is predominantly expressed in the aorta [39] or after injury to the carotid artery [23], and might play a more important role. To understand the more exact mechanism of action of ER in the vascular wall, we should make an effort to resolve this

discrepancy and are thus preparing a rat study to examine the effect of ERβ on VSMC proliferation in vivo.

Acknowledgements

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LETTERS TO THE EDITOR

THE AMERICAN GERIATRICS SOCIETY/AMERICAN ASSOCIATION FOR GERIATRIC PSYCHIATRY MENTAL HEALTH IN NURSING HOMES CONSENSUS STATEMENT

To the Editor: The National Citizens' Coalition for Nursing Home Reform (NCCNHR) had the privilege of serving on the expert panel involved in the development of two articles found elsewhere in this issue of the *Journal of the American Geriatrics Society:* the American Geriatrics Society/American Association for Geriatric Psychiatry *Recommendations for Policies in Support of Quality Mental Health Care in U.S. Nursing Homes* and the *Consensus Statement on Improving the Quality of Mental Health Care in U.S. Nursing Homes: Management of Depression and Behavioral Symptoms Associated with Dementia.* We are pleased to endorse the consensus statement but regret that the policy recommendations do not call for prompt implementation of nurse staffing ratios that experts consistently identify as necessary to provide for the basic care of nursing home residents. Although additional research might find an ideal ratio for caring for those with dementia and mental illnesses, we know from existing research that minimum needs cannot be met without at least 4.1 hours of direct care a day. Fewer than 10% of nursing homes meet this standard, and more than half fall so far below it that residents are in jeopardy. NCCNHR advocates a minimum standard of 4.13 hours—a level validated by a consensus panel of the Hartford Center for Geriatric Nursing, a congressional study of nurse staffing ratios released by the Department of Health and Human Services last year, and other research. As the policy statement says so well, "High-quality mental health care in nursing homes is possible only when the level of nurse staffing is adequate to provide the necessary amount of direct care."

Donna R. Lenhoff, Esq
National Citizens' Coalition for Nursing Home Reform
Washington, DC

RESPONSE TO DONNA LENHOFF'S LETTER ON MENTAL HEALTH CARE IN NURSING HOMES, NURSE STAFFING STUDIES

The above letter was referred to the authors of the original paper, and their reply follows.

To the Editor: The participation of the National Citizens' Coalition for Nursing Home Reform (NCCNHR), in addition to that of a broad group of organizations that reflect the multidisciplinary nature of nursing home care for frail elderly people, greatly enhanced the American Geriatrics Society/American Association for Geriatric Psychiatry Expert Panel on Quality Mental Health Care in Nursing Homes. Through its representative on the panel and considerable input from other leadership NCCNHR made major contributions to the consensus and policy documents published in this issue of the *Journal.* We believe that their participation added a unique perspective, and we thank them for their hard work and endorsement of the consensus statement.

It was the panel's concern over the adequacy of current nursing home staffing, along with several other concerns, that prompted the panel to develop a policy document along with the consensus statements. The panel felt strongly that the consensus statements themselves, without changes in health policy, would not result in meaningful improvements in mental health care in nursing homes. Although we cannot speak for the entire panel or all of the organizations represented on it, we would certainly not argue with NCCNHR's recommendation for immediate implementation of the staffing standards outlined in Ms. Lenhoff's letter in the 90% of facilities that currently do not meet these standards. Nevertheless, we believe that further research into optimal staffing, in terms of numbers and training, is necessary and will be a worthwhile investment in improving the care of the large and growing numbers of nursing home residents with depression and behavioral symptoms associated with dementia, as well as with other mental health conditions.

Joseph G. Ouslander, MD
Division of Geriatric Medicine and Gerontology
Wesley Woods Center of Emory University
Emory Center for Health in Aging
Atlanta, GA
Birmingham/Atlanta VA GRECC
Atlanta, GA

Stephen J. Bartels, MD
Department of Psychiatry
Dartmouth Medical School
Hanover, NH

TESTOSTERONE AND COMPREHENSIVE GERIATRIC ASSESSMENT IN FRAIL ELDERLY MEN

To The Editor: A low plasma testosterone level in elderly men has been implicated in various diseases, including atherosclerosis, osteoporosis, and dementia,[1] but the relationship between plasma testosterone and functional measures in frail elderly men has not been addressed. Here, we report a small-scale study in which a low plasma testosterone

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level was associated with a functional decline based on comprehensive geriatric assessment in frail elderly men without malnutrition.

Fifty-four consecutive men (aged 70–95, mean age ± standard deviation = 82 ± 6 years) attending the health service facilities for the elderly (Mahoroba-no-Sato) located in Nagano Prefecture for admission or day-care service were enrolled. Of the subjects, five were diagnosed as having dementia of the Alzheimer type, six had history of stroke, five had a history of heart failure, eight had hypertension, and five had hyperlipidemia. Subjects with malnutrition, malignancy, or endocrine disease were excluded because these diseases may affect plasma androgen level. A commercial laboratory determined plasma total testosterone (total-T) and free testosterone (free-T) in addition to blood cell counts and blood chemical parameters. Total-T and free-T were assayed using a sensitive radioimmunoassay, and the intra-assay coefficients of variation were 5% to 6% and 2% to 5%, respectively. Basic activities of daily living (ADLs) were assessed using Barthel Index, instrumental activities of daily living (IADLs) by Lawton and Brody, cognitive function using Hasegawa Dementia Scale—Revised (HDS-R), mood using the Geriatric Depression Scale (GDS; 15 items), and ADL-related vitality using Vitality Index.[2]

On average, the subjects showed mild to moderate functional decline (Table 1). Also, mean plasma levels of total-T (365 ± 172 ng/dL) and free-T (5.7 ± 2.8 pg/mL) were lower than those reported in healthy elderly men[3] but comparable with those in frail elderly men.[4] As shown in Table 1, total-T and free-T were significantly correlated with functional measures except for GDS. There was no significant correlation between total-T or free-T and age, body mass index, blood hemoglobin, lymphocyte count, serum albumin, or serum total cholesterol, probably because the subjects had good nutritional status; serum albumin was 4.1 ± 0.3 g/dL, and serum total cholesterol was 179 ± 28 mg/dL. Furthermore, multivariate analysis with age, serum albumin, and serum total cholesterol as independent variables revealed that free-T was an independent determinant for the HDS-R ($R = 0.403$, $P = .03$) and Vitality Index ($R = 0.407$, $P = .02$). In similar multivariate analyses, free-T was not an independent determinant for the Barthel Index or IADLs, and total-T was not an independent determinant for each of the functional measures (data not shown).

The present study demonstrated that a higher plasma testosterone level was associated with higher scores of comprehensive geriatric assessment except for the GDS. Free-T, the active form of testosterone, showed a stronger correlation than did total-T. It has been reported that a higher total-T was associated with better ADL performance such as transferring and eating in frail elderly men.[4] The result is consistent with ours, but free-T and nutritional assessment were not included in that report. It is known that malnutrition is associated with low ADL and low plasma testosterone. In our preliminary study in elderly male patients in sanatorium-type wards, serum albumin was correlated with the Barthel Index and plasma total-T and free-T (data not shown). Consequently, nutritional markers such as serum albumin should be included as confounding factors in a study that examines the relationship between plasma testosterone and ADLs in frail elderly men. In fact, no significant relationship was found between plasma testosterone (total-T and free-T) and ADLs (Barthel Index and IADLs) in multivariate analyses including nutritional markers, although the subjects were well nourished. Alternatively, the correlation of free-T with cognitive function and ADL-related vitality remained significant in multivariate analyses. Contrary to a previous report,[5] depressed mood did not relate to plasma testosterone in this study. The reason is unknown, but it might be due simply to the cohort difference between community-dwelling healthy men[5] and frail elderly men or to the low reliability of GDS in demented people.[2]

The detailed and causal relationships need to be examined in large-scale and longitudinal studies. Nevertheless, our results suggest that testosterone treatment might improve global function in frail elderly men with low testosterone levels.

Masahiro Akishita, MD
Shizuru Yamada, MD
Hiromi Nishiya, MD
Kazuki Sonohara, MD
Mitsuo Ohni, MD
Kenji Toba, MD
Department of Geriatric Medicine
Kyorin University School of Medicine
Tokyo, Japan

Table 1. Distribution of Functional Measures and Correlation Coefficients Between Plasma Testosterone Level and Functional Measures

Measure	Barthel Index	IADL	HDS-R	GDS	Vitality Index
Mean ± standard deviation (range)	73 ± 27 (5–100)	2.1 ± 2.0 (0–5)	18 ± 7 (2–29)	6.3 ± 3.1 (1–13)	8.8 ± 1.8 (3–10)
Total testosterone	.422†	.279*	.344*	.077	.370†
Free testosterone	.369†	.390†	.512‡	.164	.464‡

Note: The Barthel Index was used to assess Activities of daily living, Lawton and Brody's instrumental activities of daily living (IADL) index to assess IADLs, the Hasegawa Dementia Scale—Revised (HDS-R) to assess cognitive function, the Geriatric Depression Scale (GDS—15 items) to assess mood, and the Vitality Index to assess ADL-related vitality.
*$P < .05$;
†$P < .01$;
‡$P < .001$.

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ROLE OF CHOLINESTERASE INHIBITOR IN THE MANAGEMENT OF SEXUAL AGGRESSION IN AN ELDERLY DEMENTED WOMAN

To the Editor: Aggressive behavior, seen in elderly demented patients, includes hitting, grabbing, pushing, biting, kicking, scratching, and throwing objects. Sexual aggression is seen in some of these patients. Sexual activity can increase in association with progression of dementia. This can result in unreasonable and exhausting demands on sexual partners at unacceptable times and inappropriate places. Occasionally, aggression may result if these needs are not met. This challenging behavior is seen in elderly patients living in nursing homes and at home. These patients are among the most difficult to manage and present a tremendous challenge to physicians. Except for hormonal therapy in male patients, pharmacotherapy has shown minimal efficacy. We present a case report of sexual aggressiveness seen in an elderly demented woman and discuss the options for management.

CASE

The patient was a 72-year-old white female who came to the outpatient senior's clinic for cognitive and behavioral evaluation. Her behavioral problems included a 6-month history of agitation and sexual aggressiveness. According to her husband, she had been sexually passive throughout her life. During the previous 6 months, she had wanted intercourse or oral sex multiple times a day, which had caused considerable stress for the husband. Her medical history included osteoporosis and she was being treated for this with etidronate. No past psychiatric history was reported. No alcohol or recreational drug abuse was reported. There was no history of aggressiveness or sexually deviant behavior in the past.

She scored 16 of 30 on Mini-Mental State Examination and 3 of 15 on the Geriatric Depression Scale. A diagnosis of mixed dementia (Alzheimer's and vascular) of moderate severity was made after cognitive evaluation, laboratory evaluation, and neuroimaging investigations. She was started on rivastigmine 1.5 mg orally two times a day. After 4 weeks, her husband reported some improvement in the patient behavior. The dose of rivastigmine was increased to 3 mg two times a day. After an additional 4 weeks, her husband reported that her agitation had decreased and her sexual aggressiveness had subsided significantly. She continued 3 mg twice a day with no apparent adverse effects.

DISCUSSION

Most of the treatments suggested for aggressiveness in dementia have only marginal benefit for controlling sexual aggressiveness. These include drugs such as antipsychitric including the newer neuroleptics like risperidone, olanzapine, clozapine, and quetiapine and the selective serotonin reuptake inhibitors, trazodone, buspirone, lithium, and valproate.[1,2]

Sexually aggressive behavior in men has been treated with antiandrogens, estrogen, and medroxyprogesterone acetate.[3,4] One study points out that cimetidine, which has antiandrogen properties, decreases libido and hypersexual behavior without serious side effects.[5]

To the best of our knowledge, this is the first case report discussing sexual aggressiveness in a female and the use of cholinesterase inhibitor in the treatment of sexual aggressiveness. Our experience with this patient indicates that cholinesterase inhibitors may be helpful in controlling sexual aggressiveness in demented patients. There have been reports that neurotransmitters, such as dopamine, and low serotonin levels played a role in aggressive behavior. The resolution of this patient's sexual aggressiveness when treated with rivastigmine raises the possibility that neurotransmitter deficits including acetylcholine play a role in the causation of sexual aggression in demented patients.

Kannayiram Alagiakrishnan, MD, FRCP(C), ABIM
Anne Sclater, MD, FRCP(C)
Duncan Robertson, MD, FRCP (C)
From the Division of Geriatric Medicine
University of Alberta
Edmonton, Canada

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PROLONGED SURVIVAL OF AN ELDERLY WOMAN WITH SALMONELLA DUBLIN AORTITIS AND CONSERVATIVE TREATMENT

To the Editor: The incidence and the mortality of bacteremia increases with age.[1] Of all organisms causing bacteremia in patients aged 65 and older, gram-negative bacilli account for approximately 60% of cases.[2] The following is a case of a female patient who presents an infrequent complication of a gram-negative bacteremia.

CASE REPORT

An 81-year-old female patient was admitted to our geriatric department with a fever persisting for 1 month. Ischemic and hypertensive heart failure associated with type 2 diabetes mellitus characterized her medical history. One

□ ORIGINAL ARTICLE □

Effects of Oxygen Administration on the Circulating Vascular Endothelial Growth Factor (VEGF) Levels in Patients with Obstructive Sleep Apnea Syndrome

Shinji TERAMOTO, Haruki KUME*, Hiroshi YAMAMOTO, Takeo ISHII**, Akira MIYASHITA**, Takeshi MATSUSE**, Masahiro AKISHITA***, Kenji TOBA*** and Yasuyoshi OUCHI

Abstract

Objective **Repeated nocturnal hypoxia is implicated in the pathogenesis of cardiovascular complications in obstructive sleep apnea syndrome (OSAS). We hypothesized that circulating vascular endothelial growth factor (VEGF) levels are affected by nocturnal hypoxemia in patients with OSAS.**

Methods **We examined the serum VEGF levels in patients with OSAS and in control subjects. We also tested the effects of oxygen or air administration on the subjects' VEGF levels.**

Patients and Materials **Twenty-four OSAS patients (mean age 54.2±3.6 years) and 24 age-matched control subjects (53.2±3.6 years). Their serum samples were tested.**

Results **Serum VEGF levels at 8 : 00 AM were significantly higher in OSAS patients than in controls ($p<0.01$). VEGF levels decreased from 515±31 (pg/ml) to 178±16 (pg/m) ($p<0.01$) in OSAS patients whose nocturnal hypoxemia was found to be improved by administration of 2 *l*/min of oxygen during the night. However, the administration of compressed air affected neither the VEGF level nor nocturnal oxygen desaturation in OSAS patients.**

Conclusion **These results indicate that circulating VEGF levels are elevated in OSAS patients, primarily due to nocturnal hypoxemia.**

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Key words: OSAS, hypoxemia, oxygen therapy, cardiovascular complications, vascular remodeling

Introduction

Obstructive sleep apnea syndrome (OSAS) is now recognized as an important sleep disorder, contributing to excessive daytime sleepiness, cardiovascular dysfunction and the impairment of health-related quality of life (1–6). Intermittent repeated hypoxia during night, hypoxic pulmonary vasoconstriction and altered cardiovascular variability are implicated in the subsequent development of overt cardiovascular diseases, resulting in increased mortality (7, 8). There is growing evidence that patients with OSAS have an increased risk of having cardiovascular complications, such as hypertension, cardiac arrhythmia (9), myocardial infarction (10), pulmonary hypertension (11), and stroke (12). Vascular endothelial growth factor (VEGF) is a potent angiogenic factor whose expression is induced by hypoxia (13, 14). VEGF has detectable levels in circulation and its expression is highly regulated by oxygen tension. We therefore hypothesized that serum VEGF levels are elevated in OSAS patients.

The aim of the present study was to compare the serum level of VEGF between OSAS and control subjects. Additionally, in order to define the causal relationship between circulating VEGF levels and nocturnal hypoxemia in OSAS patients, we examined the effects of oxygen administration on serum VEGF levels in patients with OSAS.

Methods

Subjects

Twenty-four patients with OSAS [19 men : 5 women, mean (±SD) aged 54.2±3.6 (range 35–66)] and 24 age-

From the Department of Geriatric Medicine, *the Department of Urology, The University of Tokyo Hospital, Tokyo, **the Department of Pulmonary Medicine, Yokohama City University Medical Center, Yokohama and ***Department of Geriatric Medicine, Kyorin University, Tokyo

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Reprint requests should be addressed to Dr. Shinji Teramoto, the Department of Geriatric Medicine, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655

matched controls [20 men : 4 women, mean (±SD) aged 53.2±3.6 (range 30–68)] were studied. OSAS was defined as the presence of >10 obstructive sleep apneas or mixed apneas per hour of sleep on polysomnography in association with snoring and excessive daytime sleepiness. No subjects consumed alcohol on a regular basis, nor did they take hypnotics, sedatives, analgesics, or other medications with known effects on sleep and ventilation. No subjects had evidence of chronic obstructive pulmonary diseases or other chronic lung disease, and no evidence was found of any cardiovascular disease likely to affect pulmonary hemodynamics. Current and ex-smokers were excluded from this study, since endothelial function may be affected by smoking. The demographic data relevant to the study are shown in Table 1. Informed consent was obtained from all subjects. The study protocol was approved by the committee on ethics and the Institutional Review Board of Tokyo University Hospital.

Spirometry and expiratory flow were measured using a spirometer (CHESTAC-5v, Chest Co., Tokyo, Japan). Forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were measured. For spirometric indices, we selected the best of the three maximal flow-volume curves with the best being defined as the curve with the highest sum of FVC and FEV_1 (15).

Sleep study

For polysomnographic study, all subjects were admitted to our hospitals for two or more consecutive nights. Polysomnography consisted of 8 hours of overnight monitoring using the standard technique (16). Respiratory effort was measured by respiratory inductance plethymography (Respitrace Corp., Ardsley, NY, USA), and thermistors were used to measure airflow at the nose and mouth. Surface electrodes were applied to obtain an electroencephalogram, electrooculogram [EOG], electrocardiogram, and a record of heart rate. Arterial oxygen saturation (SaO_2) was recorded with a pulse oximeter (502-P, Criticare Systems Inc., Centrais, MO, USA). A polygraph was used to record data on both the paper of a 6-channel chart recorder (Nihonkoden Co., Tokyo, Japan) and a floppy disk via an IBM-compatible personal computer data acquisition system (NEC 9801, NEC, Tokyo, Japan). In subjects who slept for less than 6 hours as determined by EEG and EOG, repeat sleep studies were performed to assess whether poor sleep led to a missed diagnosis or an inaccurate estimation of disease severity. Apnea was defined as the cessation of oronasal airflow for more than 10 seconds, and hypopnea as a reduction of 50% or more in the oronasal flow in relation to the prevailing values during preceding normal breathing, with the reduction lasting for at least 10 seconds. For determinant of the role of apenas/hypopneas wihtout hypoxemia on the serum levels of VEGF, oxyhemoglobin desaturation was not a criterion for scoring either apnea or hypopnea. For the purpose of this study, sleep apnea syndrome (SAS) was considered to be determined by apnea+hypopnea index (AHI) values greater than 10/hour.

After one week of oxygen administration (2 *l*/min) during the night via nasal prong, sleep studies were repeated to assess the effects of oxygen supplementation on the severity of apneas and arterial oxygen desaturation during the night in patients with OSAS. As a control study, the same group of OSAS patients also received compressed air for one week during the night via nasal prong. Sleep studies were then repeated to assess the effects of air administration on the VEGF levels, severity of apneas and arterial oxygen desaturation during the night in patients with OSAS. Oxygen and air were randomly administered using a cross-over protocol with a 1-week washout period.

Quantification of VEGF concentration in serum

Peripheral blood samples were obtained from all OSAS patients at 8:00 AM and 8:00 PM on the day prior to administration of oxygen or compressed air, and on the day after 1-week of oxygen or compressed air administration. The samples were immediately centrifuged for 10 minutes at 3,000 rpm. The supernatant fluid was stored in a refrigerator at –70°C for later analysis. Serum levels of VEGF were determined by a commercially available ELISA test (R&D Systems, Wiesbaden, Germany). This assay, which measures free, unbound VEGF, is calibrated against a highly purified recombinant human $VEGF_{165}$, the major and most potent isoform of VEGF. The VEGF assay had a minimum sensitivity of 9 pg/ml, with intra-assay coefficients of variation of 6.7% and 4.5% for concentrations of 54 and 235 pg/ml, respectively. All VEGF measurements were carried out on the same day after completion of all sleep studies.

Statistical analysis

The Mann-Whitney nonparametric test was used to compare the variables of demographic data and pulmonary function data in both OSAS patients and control subjects. An analysis of variance with 1 fixed factor with repeated measures was used to compare the variables baseline, air and oxygen and 2 subject groups. The associations of serum VEGF levels with other variables were tested using linear regression analysis. All analyses were performed by a software package using Stat View 5.0 (Abacus Concepts, Inc., Berkeley, CA, USA). The data are presented as mean±SD. $p<0.05$ was considered to be statistically significant.

Results

In the current study, all participants were obese, both in the OSAS group [body mass index (BMI)=29.0±1.6 (kg/m^2)] and in the control (CTRL) group [BMI=28.6±1.7 (kg/m^2)]. Spirometric indices were within the normal range in all subjects. Anthropometric and pulmonary function data are shown in Table 1. In the OSAS group, all 24 OSAS patients had moderate to severe obstructive sleep apnea (i.e., obstructive or mixed apneas/hypopneas per hour of sleep were greater than 10) and their mean AHI was 38.6±4.8. In the CTRL group, no subject showed more than 5 apneas/

Table 1. Demographic and Anthropometric Data

	OSAS	CTRL
Number of subjects	24	24
Male : Female	19 : 05	20 : 04
Age (years old)	54.2±3.6	53.2±3.6
Height (cm)	164±6	162±5
Weight (kg)	78±6	75±6
BMI (kg/m^2)	29.0±1.6	28.6±1.7
FVC (*l*)	2.82±0.26	2.79±0.22
FEV_1(*l*)	2.42±0.12	2.38±0.11
FEV_1/FVC (%)	85.8±5.6	85.3±5.1
PaO_2 (mmHg)	68.2±2.1	70.2±1.8
$PaCO_2$ (mmHg)	42.2±1.3	41.8±1.2
SBP (mmHg)	138±4.8	132±4.6
DBP (mmHg)	74±2.4	73±2.2

Data were presented as mean±SD. OSAS: obstructive sleep apnea syndrome, CTRL: control subjects without OSAS, BMI: body mass index, FVC: forced vital capacity (*l*), FEV_1: forced expiratory volume in one second (*l*), PaO_2: arterial pressure of oxygen, $PaCO_2$: arterial pressure of carbon dioxide, SBP: systolic blood pressure, DBP: diastolic blood pressure.

hypopneas per hour, and their AHI was 1.8±0.8 (Table 2). Although the baseline value of SaO_2 in patients with OSAS was nearly identical to that in the control subjects, the nadir SaO_2 values were considerably lower in patients with OSAS than in control subjects ($p<0.01$). While the occurrence of apnea during sleep in patients with OSAS was not significantly reduced by the administration of oxygen, the rate of 4% arterial oxygen desaturation from baseline SaO_2 in our patients was markedly reduced by oxygen administration (Table 2). Both nadir SaO_2 and mean SpO_2 during night were improved by oxygen supplementation, but not by the administration of compressed air. In control subjects without OSAS, oxygen administration (2 *l*/min) did not affect the number of apneas or the nadir SaO_2 (Table 2).

The serum VEGF level (515±31 pg/ml) after sleep was higher ($p<0.01$) in OSAS patients than in control subjects (152±13 pg/ml). The value before sleep, taken at 8:00 PM, was also higher in OSAS patients than in control subjects ($p<0.01$) (Table 3). After oxygen administration, VEGF levels in OSAS patients both before and after sleep decreased significantly. However, the administration of air had no effect on their serum VEGF levels. In control subjects, the administration of neither oxygen nor air affected the serum

Table 2. Effects of the Supplementation of Oxygen or Compressed Air on the Numbers of Apneas and Arterial Oxygen Desaturation in OSAS Patients and Control Subjects

Supplementation	OSAS			CTRL		
	Non	Air	Oxygen	Non	Air	Oxygen
Number of subjects	24	24	24	24	24	24
AHI (/hour)	39±5*	36±6*	35±6*	2±1	2±2	2±2
Baseline SaO_2 (%)	95±2	96±1	95±2	95±2	96±2	97±2
Nadir SaO_2 (%)	70±8*	73±4#	90±3	93±2	93±2	95±1
N of 4% desaturation	264±5*	246±7*#	28±6*	4±2	3±2	2±2
Mean SaO_2 (%)	89±2*	90±1*#	93±1	94±1	94±2	95±1

Data were presented as mean±SD. OSAS: obstructive sleep apnea syndrome, CTRL: control subjects without OSAS, Air: 1–2 ml/min of compressed air was administered during the night via nasal prong for a week. Oxygen: a week oxygen administration (2 *l*/min) during the night via nasal prong. AHI: apnea and hypopnea index, Baseline SaO_2: stable value of SaO_2 at supine position before sleep (%), Nadir SaO_2: the lowest value of SaO_2 during night (%), N of 4% desaturation: number of 4% of arterial oxygen desaturation from baseline value of SaO_2, Mean SpO_2: the mean value of SaO_2 during night (%). *$p<0.01$ compared with the same value of CTRL, #$p<0.01$ compared with the same value without oxygen supplementation.

Table 3. Serum Levels of VEGF before and after Sleep Studies

Supplementation	OSAS			CTRL		
	Non	Air	Oxygen	Non	Air	Oxygen
VEGF before sleep (μM)	457±26*	469±29*	165±12#	161±23	158±13	145±16
VEGF after sleep (μM)	515±31*	526±34*	178±16#	152±13	165±22	150±14

Data were presented as mean±SD. Air: 2 ml/min of compressed air was administered during the night via nasal prong for a week. Oxygen: one week of oxygen administration (2 *l*/min) during the night via nasal prong. *$p<0.01$ compared with the same value of CTRL, #$p<0.01$ compared with the same value without air/oxygen administration.

Table 4. Relationships between the Serum Levels of VEGF and Other Variables in All subjects

	p	r
AHI (/hour)	0.05	r= 0.401
Baseline SaO_2 (%)	0.27	r=–0.223
Nadir SaO_2 (%)	0.01	r=–0.457
N of 4% desaturation	0.01	r= 0.581
Mean SaO_2 (%)	0.05	r=–0.389
Systolic BP	0.34	r= 0.027
Diastolic BP	0.41	r= 0.123

BP: blood pressure.

VEGF level (Table 3).

In examining the relationship between VEGF levels and numerous of parameters (AHI, nadir SaO_2, systolic blood pressure, diastolic blood pressure, arterial oxygen desaturation), we found that there were significant relationships between VEGF levels and AHI, nadir SaO_2, mean SpO_2, and the number of 4% oxygen desaturation (Table 4).

Discussion

The present study demonstrates that the administration of 2 *l*/min oxygen during the night to OSAS patients improves nocturnal hypoxemia in parallel with a decrease in VEGF levels. Serum VEGF levels were greater in obese patients with OSAS than in obese control subjects. Therefore, we conclude that circulating VEGF levels are elevated in OSAS patients, primarily due to nocturnal hypoxemia. Nevertheless, numerous factors might influence VEGF serum levels and must be taken into consideration when discussing these observations. The most common conditions known to be associated with elevated VEGF serum levels, such as disseminated cancer and chronic inflammatory and autoimmune disease, were not present in either the patients or the control subjects in the current study (17).

It has been reported that serum VEGF levels are significantly higher in patients with polysomnographically confirmed OSAS (AHI>15 and AI>5 in adults and children, respectively) than in patients with mild disease or no disease (18, 19). Furthermore, significant correlations have been found between VEGF concentrations and respiratory disturbance index and sleep time spent at SpO_2 <90% (18). Schulz et al reported that serum levels of VEGF are elevated in severely hypoxic patients with OSAS and are related to the degree of nocturnal oxyhemoglobin desaturation (20). They speculated that the most likely trigger of VEGF release in OSAS is hypoxia, since a close linear relationship between the degree of nocturnal oxyhemoglobin desaturation and VEGF concentrations was observed (20). If this is true, the administration of oxygen to patients with OSAS could reduce their VEGF levels to the normal levels found in subjects without OSAS. In the present study, we found that nighttime administration of oxygen, but not compressed air, decreased the VEGF level and improved nocturnal oxyhemoglobin desaturation, but did not affect the occurrence of nocturnal apneas. It is reasonable to assume that repeated episodes of nocturnal hypoxemia are the primary mechanism of the increased production of VEGF in patients with OSAS.

However, previous studies have suggested that AHI is correlated with VEGF levels in OSAS patients (20). Because the administration of oxygen to OSAS patients improves both VEGF levels and nocturnal hypoxemia but not AHI, the relationship between AHI and VEGF levels may be associated with AHI-related nocturnal hypoxemia, but not directly with the number of apneas/hypopneas.

Other mediator systems related to hypoxia may also be involved in the mechanism of VEGF increase in patients with OSAS. Because reactive oxygen species and endothelin are elevated in OSAS patients (21, 22), these mediators may enhance gene expression of VEGF (23, 24). Because nitric oxide synthesis is downregulated in OSAS patients (25, 26), the inhibitory effect of nitric oxide on VEGF gene induction could be weakened in these patients.

The clinical significance of elevated VEGF concentrations in patients with sleep apnea remains a matter of speculation at this stage. VEGF is a mitogen specific for endothelial cells, which appears to play a pivotal role in physiological and pathological angiogenesis (13). VEGF has been shown to be an important factor in the pathogenesis of vascular-related diseases, including the growth of tumors, vascular dysfunction in diabetes mellitus, and atherosclerosis of the coronary arteries (13, 27, 28). The increased VEGF production found in OSAS patients might contribute to new vessel formation in ischemic and atherosclerotic vascular regions. Thus, enhanced VEGF production may constitute an adaptive mechanism to counterbalance the emergence of cardiovascular disease in patients with OSAS. The augmented VEGF concentration in sleep apnea patients having comorbidities may reflect a contributory mechanism for the development of cardiovascular disease. Recent data have suggested that amelioration of nocturnal hypoxia by nasal continuous positive airway pressure (nCPAP) is associated with a significant decrease in morning VEGF concentrations (29). The alterations of the VEGF system in patients with OSAS may have an impact on the development of cardiovascular abnormalities in these patients. Therefore, a reduction of VEGF concentration following treatment of sleep apnea and potentially reduced angiogenesis may be a clinically significant goal of treatment.

In conclusion, serum levels of VEGF are elevated in patients with OSAS and are closely correlated with the degree of nocturnal oxyhemoglobin desaturation. This increased VEGF was decreased by nighttime oxygen administration in OSAS patients, suggesting that nighttime hypoxia is a primary mechanism of VEGF increase in these patients. However, further studies are necessary to explore the clinical significance of VEGF increase on outcome in terms of cardiovascular diseases and mortality in OSAS patients.

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Classification of the dementias

Sir—Alistair Burns and Michael Zaudig (Dec 14, p 1963)[1] postulate a range of cognitive impairments between normal ageing and dementia in older people. We think that there might be a pathological stage in individuals with subjective memory complaints but who do not meet the current criteria for the diagnosis of mild cognitive impairment (MCI). We have seen two patients with increased concentrations of cerebrospinal fluid tau (CSF-tau) protein and subjective memory complaints alone.

The first was a 72-year-old hypertensive man who had noticed a subtle cognitive change over the previous 6 months. He complained of difficulty in retrieving recent personal events and in planning his travel schedule. There were a few lacunes on MRI, with no noticeable neurological deficits. A brain perfusion study with (123I)-iodoamphetamine (IMP) single-photon emission CT (IMP-SPECT) was unremarkable. He scored 28 points on the mini mental state examination (MMSE), 10·7 points on the Alzheimer's disease assessment scale (ADAS-cog), and 7 points on the geriatric depression scale (GDS). There was no decline in scores of the delayed recall subscales of the revised Wechsler memory scale after adjustment for age. CSF-tau concentration was 351·1 ng/L, which was in the Alzheimer's disease range. His memory failure was progressive, and he finally met the criteria for MCI a year later.

The second patient was a 76-year-old woman who noticed an insidious memory impairment over the previous year. She scored 29 points on MMSE, 8·3 points on ADAS-cog, and 8 points on GDS. There was no decline in scores of the delayed recall subscales of the revised Wechsler memory scale after adjustment for age. Both MRI and IMP-SPECT were unremarkable. The CSF-tau concentration was 400·2 ng/L, which was also in the range compatible with Alzheimer's disease. She continued to feel declining memory function, but did not reach the criteria for MCI until recently.

CSF-tau is a sensitive indicator that could reflect pathological accumulation of tau and overall neuron death.[2] Although not all individuals who complain of subjective memory loss eventually develop Alzheimer's disease, we have previously shown that increased CSF-tau concentrations are highly predictive of progressing from MCI to Alzheimer's disease.[3] The present observation might provide evidence for prodromal stages between cognitive change and overt MCI.

*Hiroyuki Arai, Toshifumi Matsui, Masahiro Maruyama, Nobuyuki Okamura, Hidetada Sasaki

Department of Geriatric Medicine, Tohoku University School of Medicine, Sendai, Miyagi 981-8574, Japan (e-mail: h-ara@mail.cc.tohoku.ac.jp)

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