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Dose-dependent effect of dehydroepiandrosterone, but not of its sulphate ester, on angiogenesis

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Abstract

Although dehydroepiandrosterone (DHEA) is widely used in the elderly to prevent some adverse effects of ageing, possible deleterious side effects have not been fully assessed. We evaluated the direct actions of DHEA and DHEA sulphate on angiogenesis, a critical event in pathologies that are common in the elderly (cancer, atherosclerosis, inflammation... etc.). At physiological concentrations found in human plasma following DHEA therapy (1–50 nM), DHEA had no action on angiogenesis in vitro. In contrast, higher concentrations of DHEA (10–100 μM), which can be found in tissues after local administration or storage, inhibited in vitro endothelial cell proliferation (blockage in G2/M), migration and capillary tube formation and in vivo angiogenesis in the Matrigel plug assay. This inhibition might be due to a decreased glucose-6-phosphate dehydrogenase activity and to a modification of the tubulin network involved in cell proliferation and migration. The sulphate ester form of DHEA had no effect on angiogenesis.

Keywords: Dehydroepiandrosterone; Dehydroepiandrosterone sulphate; Angiogenesis; Endothelial cell; Tubulin; Glucose-6-phosphate dehydrogenase

1. Introduction

Dehydroepiandrosterone (DHEA) and its sulphate ester (dehydroepiandrosterone sulphate or DHEAS) are the most abundant steroids produced by the adrenal cortex in humans. Plasma levels of DHEA are in the nanomolar range (up to 50 nM), whereas those of DHEAS, the major circulating form of DHEA, are in the micromolar range (≥10 μM). Steroid sulphotransferases and sulphatases are widely distributed in the body and catalyse the metabolic conversion of DHEA and DHEAS. DHEA is also a precursor of biologically active steroids such as testosterone and estrogens (Labrie et al., 1998). However, DHEA(S) (a term used here to designate both DHEA and its sulphate ester) can display effects on its own. Studies of DHEA(S) secretion in the blood have shown that it reaches a peak value between 20 and 30 years of age and declines steadily (2% per year) in the following decades to less than 20% of the maximum after 70 years (Orentreich et al., 1984). DHEA supplementation in the elderly has therefore been proposed to restore DHEA(S) blood levels and consequently reverse or prevent adverse effects associated with ageing. The administration of DHEA to humans has been reported

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to stimulate the immune system and to exert beneficial effects on skin, muscle and bone (Baulieu et al., 2000; Legrain et al., 2000). However, possible deleterious side effects following DHEA replacement have not been extensively assessed so far.

The induction of angiogenesis is potentially dangerous because it supplies growth factors for tumour growth and allows cancer cell dissemination (Folkman, 1995). Since the aggressiveness of cancers can be greatly increased when angiogenesis is stimulated (Pili et al., 1994), a major question is raised regarding the frequency of cancer in the elderly with a low proliferative index and invasion. Angiogenesis has also been implicated in several non-neoplastic diseases that are observed in the aged population, such as the destabilisation of atheromatous plaques, leading to their rupture, which is responsible for acute ischaemic events (McCarthy et al., 1999; Moulton, 2001), and ocular neovascularisation, which is responsible for macular degeneration, a frequent cause of blindness in the elderly (Ambati et al., 2003). Furthermore, angiogenesis is also a critical process in numerous inflammatory diseases, i.e. arthritis (Carmeliet, 2003).

Angiogenesis is known to be differentially regulated by certain steroids, i.e. positively by 17β-estradiol and negatively by testosterone and 2-methoxyestradiol (Banerjee et al., 1997; Folkman and Ingber, 1987; Fotsis et al., 1994; Lansink et al., 1998; Ma et al., 2001).

The present study was aimed at investigating the actions of DHEA(S) on angiogenesis. For this purpose, two ranges of steroid concentrations were tested on human microvascular endothelial cells (HMEC-1). The lower concentrations, the physiological ones, were 1–50 nM for DHEA and 1–10 μM for DHEAS, and they correspond to the plasma concentrations of DHEA(S) that have been found in subjects orally administered 50 mg DHEA/day for 6 months to 1 year (Baulieu et al., 2000; Morales et al., 1994). Steroids were also tested at higher micromolar concentrations, i.e. 1–100 μM for DHEA and 10–100 μM for DHEAS. The rationale for investigating the effects of high micromolar steroid concentrations was based on the following: (i) high concentrations of DHEA(S) could be reached when it is locally applied or stored in a tissue (Kroboth et al., 1999; Massobrio et al., 1994). Indeed, DHEA has previously been applied in the vagina (150–300 mg DHEA per vagina), to prevent vaginal atrophy (Casson et al., 1996); and (ii) the blood steroid concentration is not a good indicator of steroid concentrations in tissues. Interestingly, the ratio of adipose tissue to serum concentrations has been shown to be the highest for DHEA, whose concentration reached 10 μM in the adipose tissue of untreated patients (Feher and Bodrogi, 1982; Feher et al., 1976). Since steroid accumulation could be of critical importance in steroid-responsive tumours and because angiogenesis is implicated in the proliferation and dissemination of several types of cancerous cells (Abulafia et al., 1999; Folkman, 2002; Sivridis, 2001), it was appropriate to examine the effects of high micromolar concentrations of DHEA on the proliferation, migration and capillary tube formation of HMEC-1.

2. Materials and methods

2.1. Chemicals

Recombinant angiogenic factors, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and oncostatin M were supplied from R&D Systems (Abington, UK). DHEA was purchased from FLUKA and DHEAS from Sigma-Aldrich (St. Quentin-Favallier, France). [4-^{14}C]-DHEA (55.2 mCi/mmol) was from New England Nuclear (Boston, MA); Δ5-androstene-3β,17β-diol (ADIOL) was generously donated by Roussel-Uclaf (Romainville, France). Analytical grade solvents were supplied by Merck (Darmstadt, Germany) and Carlo Erba (Milan, Italy). DHEA and ^{14}C-DHEA were dissolved in ethanol and DHEAS in methanol. The final concentration of ethanol or methanol in the culture medium did not exceed 1% in the control culture medium and in cultured cells treated with DHEA(S).

2.2. Endothelial cell culture

HMEC-1 were provided by Dr. Ades (Centers for Disease Control and Prevention, Atlanta, GA), who established this cell line by transfecting human dermal endothelial cells with SV40 A gene product and large T-antigen. These cells have properties similar to those of the original primary cell culture (Ades et al., 1992). HMEC-1 were used because they are representative of microvascular endothelial cells (Bouis et al., 2001), and because angiogenesis occurs in the microvasculature, but not in large blood vessels (Klagsbrun and Folkman, 1990). HMEC-1 were used before 15th passage, a condition under which HMEC-1 are sensitive to angiogenic factors. HMEC-1 were cultured in a complete medium containing MCDB-131 medium (Sigma) supplemented with 15% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml epidermal growth factor (Euromedex, Souffelweyersheim, France) and 1 μg/ml hydrocortisone (Pharmacia-Upjohn, St. Quentin-en-Yvelines, France).

2.3. Endothelial cell proliferation assay

For the cell proliferation assay, epidermal growth factor was omitted and 7.5% of fetal calf serum was used. Briefly, after trypsinisation (0.05% trypsin w/v, Sigma), HMEC-1 were seeded at a density of 5×10^4 cells per well in a 24-well plate (Nunc, Roskilde, Denmark) and incubated without or with angiogenic cytokines (2.5 ng/ml oncostatin M, 25 ng/ml bFGF, 20 ng/ml VEGF). DHEA or DHEAS was added at different concentrations, indicated in Results. After a 3-day

incubation, cells were harvested with trypsin, resuspended in Isoton II solution (Coulter, France) and counted in a particle counter (Coulter Z1, Coultronics, Margency, France).

2.4. Cytotoxicity and cell cycle analysis

For cytotoxicity studies, cells were cultured and treated with DHEA or DHEAS at 100 μM for 3 days. Cells were then collected and cytotoxicity was further assessed by Trypan blue dye exclusion.

For cell-cycle analysis experiments, cells were harvested using cell dissociation solution (Sigma) and pelleted by low-speed centrifugation. Then, cells were fixed with ethanol 70% in phosphate-buffered saline (PBS), for 30 min, at 4 °C and then washed twice in PBS. Cells, resuspended in 1 ml PBS, were incubated with 20 μg/ml of Hoechst 33342 and DNA content was analysed by flow cytometry.

2.5. "*Wound*" repair by endothelial cells

Endothelial cells were cultured in a 24-well culture plate. When HMEC-1 cells were confluent, a "wound" was made. After being washed with PBS, cells were incubated with MCDB-131 containing 2% fetal calf serum (concentration of fetal calf serum which allows cell survival but not cell proliferation) with or without the angiogenic cytokines and in the absence or presence of different concentrations of DHEA or DHEAS. After 48 h of incubation, cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS, for 10 min, at room temperature. The cells were then stained with Giemsa, and cell repair was observed and photographed with a camera connected to an inverted microscope, at 25× magnification.

2.6. Capillary tube formation in a three-dimensional fibrin gel

Microcarrier cell culture was performed according to the method of Nehls and Drenckhahn, (1995). In brief, HMEC-1 were allowed to attach to the cytodex-3 microcarrier beads (Sigma). The beads were then embedded in a fibrin matrix, obtained by coagulation of a solution of purified fibrinogen at 8 mg/ml (Kabi, Sweden) in Medium-131 (Cascade Biologics, Portland, OR) containing: bFGF (25 ng/ml) and 5% microvascular growth supplement (Cascade Biologics) as source of growth factors, 10% fetal calf serum, 1% L-glutamine and 0.2 mM aprotinin, in the presence or absence of DHEA or DHEAS. After addition of thrombin (2 units/ml, final concentration), fibrin gel was formed, and then 500 μl of complete culture medium containing 10% fetal calf serum and 5% microvascular growth supplement was added and changed every 3 days. The formation of capillary tubes arising from the periphery of microcarrier beads was observed. These capillaries were photographed with a camera connected to an inverted microscope, at 20× magnification.

2.7. Cytoskeleton analysis

2.7.1. Confocal microscopy analysis of actin filaments

Confocal microscopy analysis of actin filaments was performed according to the protocol of Menager et al., (1999) (Vincent et al., 2001), on bFGF-stimulated HMEC-1, after a 24-h incubation with DHEA (0.1, 1, 10 or 100 μM) or vehicle alone. Actin filaments were detected by staining with tetramethyl rhodamine isothiocyanate (TRITC)-labelled phalloidin. Computer-assisted image analysis of fluorescence was carried out using a confocal microscopy scanning laser microscope (Leica TCS, excitation λ=540 nm, emission λ=570 nm for TRITC).

2.7.2. Immunofluorescence microscopy of β-tubulin

HMEC-1 were grown in an eight-well Lab-Tek coverglass chambers (10^4 cells per well), previously coated with 0.2% gelatin. Cells were allowed to attach for 24 h, then medium was replaced by the one used for proliferation assays, and cells were incubated with bFGF (25 ng/ml) without or with DHEA (10, 50 or 100 μM). After a 2-day incubation, cells were washed once in PBS and fixed in a solution of PBS containing 0.2% glutaraldehyde and 4% paraformaldehyde, for 20 min, at room temperature. Cells were washed twice with a solution of PBS containing 0.5% bovine serum albumin. The fixed cells were then incubated for 15 min in PBS with 0.25% Triton X-100. Slides were incubated in a blocking solution of PBS–0.5% bovine serum albumin for 15 min, at 4°C. Cells were then incubated for 3 h at 4°C with a mouse monoclonal antibody against β-tubulin (diluted at 1:200) (Sigma). After three washes with PBS–0.5% bovine serum albumin, cells were incubated for 2 h at room temperature with an anti-mouse immunoglobulin G fluorescein isothiocyanate-conjugated antibody (DAKO) (diluted at 1:100). After three washes, the slides were mounted with cover slips and examined under a fluorescence microscope (Zeiss, Jena, Germany).

2.8. Activity of glucose-6-phosphate dehydrogenase (G6PDH)

Cells were seeded in a 75 cm^2 culture flask and grown in the same medium as that used for proliferation assays. Cells were incubated with vehicle or with DHEA at the concentrations indicated in Results, in the absence or in the presence of bFGF (25 ng/ml). After a 48-h incubation, cells were washed with PBS, then scraped off and collected in 10 ml PBS. Cells were centrifuged (10 min, 3000 rpm), resuspended in 700 μl 1% Triton X-100/PBS solution and frozen at −80 °C. G6PDH activity was determined by the

generation of NADPH from NADP during glucose-6-phosphate oxidation. Sample (50 μl) was added to 1 ml of 80 mM Tris buffer, pH 8.6 containing $MgCl_2$ $6H_2O$ (40 mM) and NADP (400 μM). Glucose 6 phosphate (300 μM) and 6 phosphogluconate (100 μM) were added. Enzyme activity was measured at 25 °C, for 5 min, by evaluating the rate of increase of absorbance at 340 nm, in a spectrophotometer (UVIKON 922).

2.9. Metabolism of ^{14}C-DHEA by endothelial cells in vitro

HMEC-1 were seeded in a 75 cm^2 culture flask and grown in MCDB-131 medium, supplemented with 7.5% fetal calf serum. ^{14}C-DHEA metabolism was analysed as previously described (Akwa et al., 1993). Briefly, when cells had reached confluency, they were incubated with 100 nM ^{14}C-DHEA in the absence or the presence of bFGF (25 ng/ml), in a final volume of 10 ml of culture medium. Control incubations were performed with culture medium alone. All incubations were carried out at 37 °C for 24 h. Media were then collected and cells were scraped off, resuspended in 5 ml PBS and counted in a particle counter (Coulter Z1, Coultronics). Steroids were extracted from media, three times with one volume of ethyl acetate. Extracted steroids from the organic phase were applied to silica gel F254 thin-layer chromatography (TLC) plates (Merck) and developed once in the solvent system chloroform/ethyl acetate 4:1 (v/v). Authentic reference Δ5-androstene-3β,17β-diol (ADIOL) was run on a separate lane and located by spraying the plates with a solution of premuline (Sigma) in 50% acetone (w/v). The Rf value of ADIOL was 0.28. Autoradiography of chromatograms was performed by exposure of Kodak Biomax XLS X-ray films (Kodak, Rochester, NY) for 5 days to locate radioactive areas. The relative amounts of ^{14}C-steroids were measured by scanning the thin-layer plates with an automatic TLC linear analyser (Multitrace model LB-285, Berthold Analytical Instruments, Nashua, NH). Other radioactive measurements, i.e. for recoveries after incubation and extraction, were carried out in 5 ml of scintillation liquid (Picofluor 15, Packard Bioscience, Groningen, The Netherlands) with a Tricarb 2100TR (Packard Instruments, Warrenville, RI) equipped with quench correction. Metabolic conversion rates are given as mean±S.E.M from one experiment in triplicate.

2.10. In vivo angiogenesis assay

To analyse the action of DHEA on in vivo angiogenesis, the Matrigel model described by Passaniti et al., (1992) was used. Briefly, 300 μl of Matrigel (11.7 mg/ml, phenol red-free, Becton Dickinson, France) maintained in liquid form (kept at 0 °C) was mixed with or without 1 μg of bFGF, with vehicle alone or with DHEA (100 μg), and then injected subcutaneously in the dorsa of 7-week-old Swiss nu/nu female mice (Janvier, Le Genest Saint Isle, France), using a 24-gauge needle. Each group contained five mice. Ten days after injection, tissue containing the Matrigel plugs, including the adjacent skin, was removed, fixed overnight in absolute ethanol and embedded in paraffin. Matrigel sections (5 μm) were prepared. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 min. Neovessels were visualised by incubating Matrigel sections with a rat antibody against mouse platelet–endothelial cell adhesion molecule 1 (Pharmingen, France) and then with a biotinylated goat anti-rat immunoglobulin G antibody. After being washed, sections were incubated with streptavidin-peroxidase and vessels were revealed with the peroxidase substrate diaminobenzidine. Meyer's haematoxylin was used for counterstaining. The vascularisation level around the Matrigel plugs was evaluated as described previously by Weidner et al., (1991) and expressed by the angiogenic index.

2.11. Statistical analysis

Results are expressed as mean±S.E.M and analysed by using a two-tailed non-parametric Mann–Whitney test using the InStat software (Sigma). Significance was defined at $P<0.05$.

3. Results

3.1. Effect of DHEA(S) on endothelial cell proliferation

DHEA, at physiological concentrations (1–50 nM), induced a slight but non-significant increase in the proliferation of unstimulated HMEC-1 and was ineffective on oncostatin M- or bFGF-stimulated HMEC-1 (Fig. 1A). At higher concentrations, a significant inhibition of the proliferation of angiogenic factor-stimulated cells was observed at concentrations from 10 μM DHEA (Fig. 1B). At 50 and 100 μM DHEA, endothelial cell proliferation was inhibited regardless of whether the cells were stimulated or not by angiogenic factors. This inhibition did not result from a cytotoxic effect, as assessed by staining with Trypan blue, compared to control (data not shown).

The effect of DHEA on the cell cycle was then analysed. In bFGF-stimulated cells in the absence of DHEA, the percentage of cells in the different phases of the cell cycle was not significantly modified by DHEA up to 10 μM. In contrast, when cells were incubated with 50 μM DHEA, an arrest of cell cycle in G2/M phase was evident after 3 days of incubation (in control cells: 35±10% of cells were in G1 phase, 55±13% in S phase and 10±4% in G2/M phase, while in cells treated with 50 μM DHEA: 14±2% of cells were in G1 phase, 19±7% in S phase and 66±8% in G2/M phase). This arrest in G2/M phase was not observed after a shorter incubation (24 h) with DHEA at the same

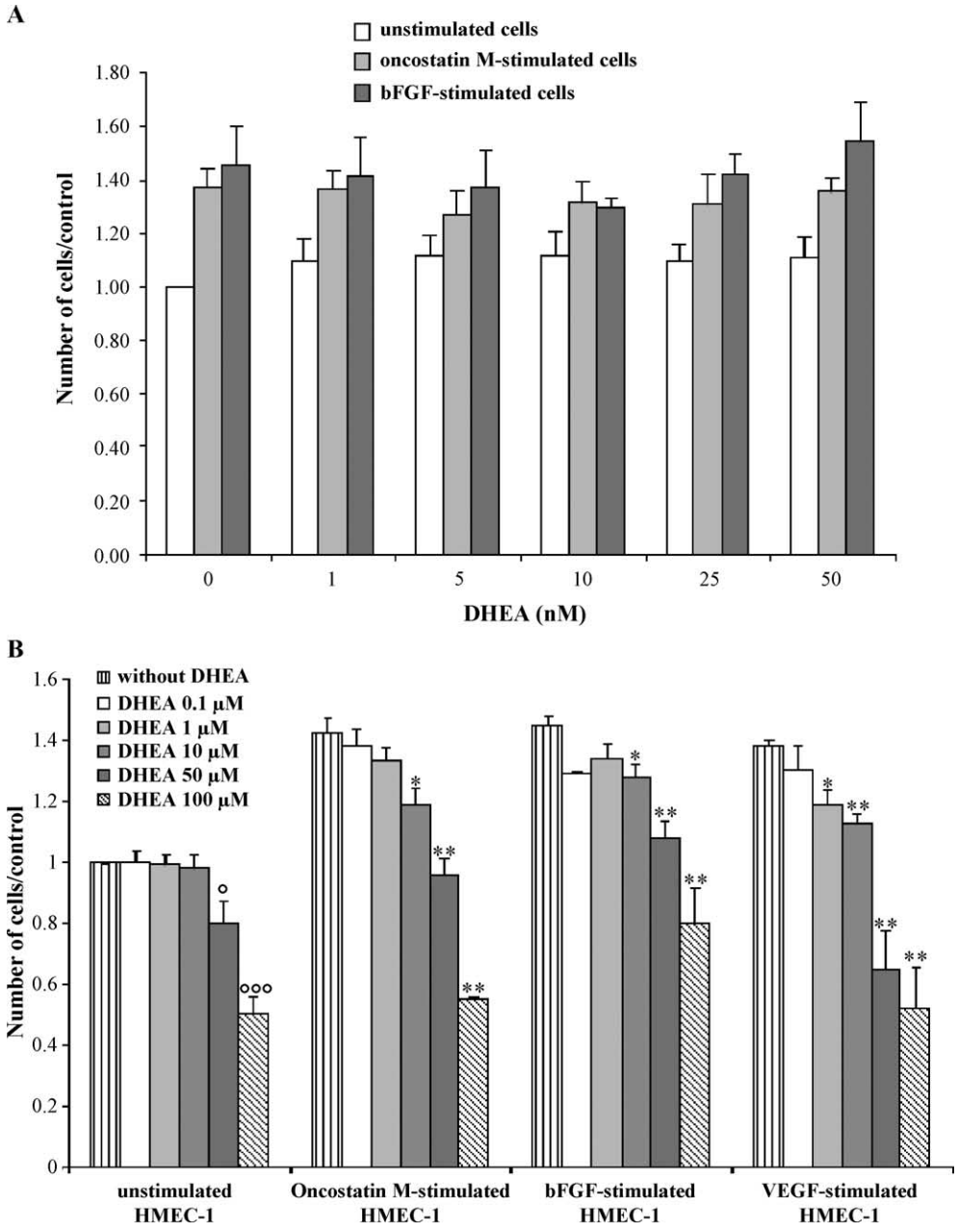

Fig. 1. Dose–response study of DHEA on endothelial cell proliferation. HMEC-1 cells, seeded at 5.10^4 cells per well, were incubated at the indicated concentrations of DHEA, in the absence or presence of angiogenic factors oncostatin M (2.5 ng/ml), bFGF (25 ng/ml) or VEGF (20 ng/ml), for 3 days. (A) Effect of physiological DHEA concentrations. Data are expressed as the ratio of the number of treated cells to the number of vehicle-treated control cells (without DHEA, without angiogenic stimulation), i.e. mean±S.E.M. from four independent experiments in duplicate. (B) Effect of pharmacological DHEA concentrations. Data are expressed as the ratio of the number of treated cells to the number of vehicle-treated control cells (without DHEA, without angiogenic stimulation), i.e. mean±S.E.M. from four independent experiments in duplicate. °P<0.05, °°P<0.01 and °°°P<0.001 compared with untreated unstimulated cells, *P<0.05, **P<0.01 and ***P<0.001 compared with oncostatin M or bFGF or VEGF-stimulated cells in the absence of DHEA.

concentration (data not shown). No significant increase in apoptotic cells was observed in the presence of high concentrations of DHEA, even after 3 days of incubation (data not shown).

Studies indicated that, at both physiological (1–10 μM) and pharmacological (50 and 100 μM) concentrations, DHEAS had no effect on the proliferation of HMEC-1, whether or not cells had been stimulated with angiogenic factors (data not shown).

3.2. Effect of DHEA(S) on endothelial cell repair

High concentrations of DHEA (50 and 100 μM) induced a strong and significant dose-dependant inhibition of "wound" repair in both unstimulated cells and cells treated with angiogenic factors (oncostatin M, bFGF or VEGF) (Fig. 2A). DHEA (1–50 nM) and DHEAS (1–100 μM) did not modify repair by HMEC-1 (data not shown).

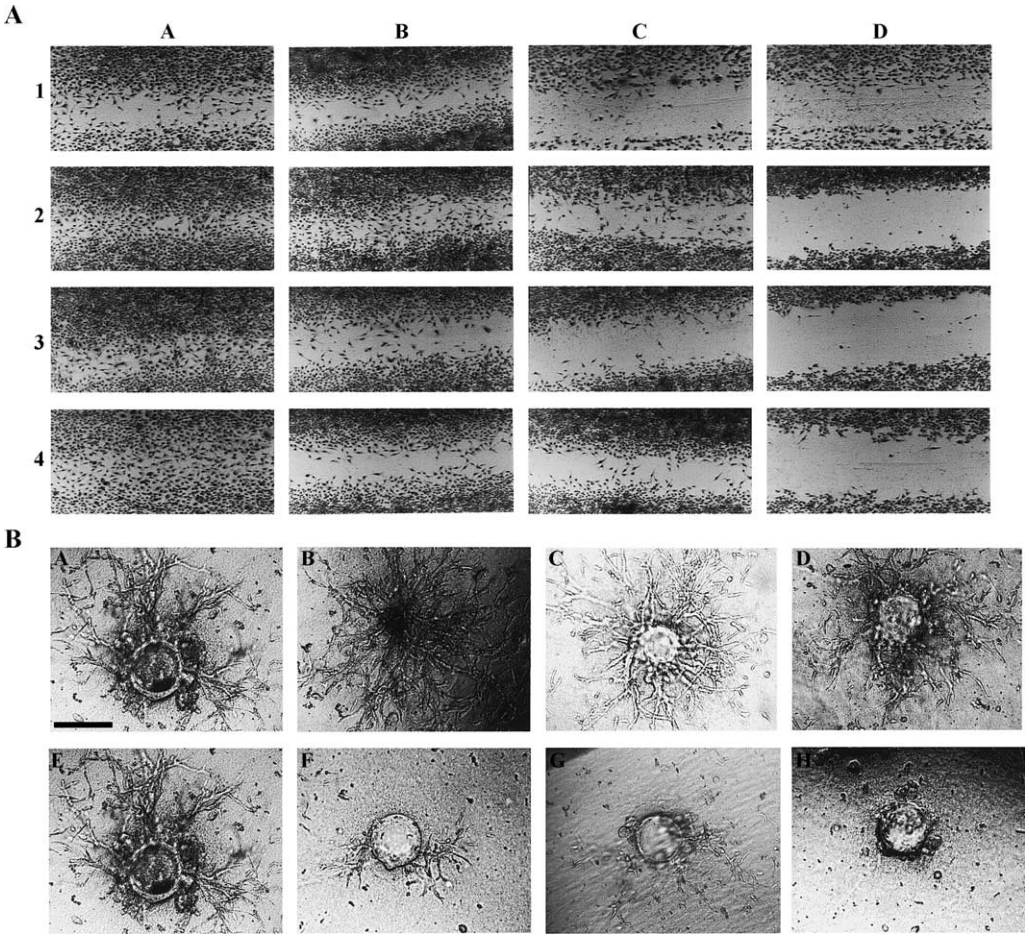

Fig. 2. Effect of DHEA on endothelial cell migration and on capillary tube formation. (A) Effect of DHEA on endothelial cell migration in the wound-healing model. Inhibitory effect induced by DHEA on unstimulated or oncostatin M-, bFGF- or VEGF-stimulated HMEC-1 migration, is shown in a wound-healing model, after a 48-h incubation (magnification ×25). Column A: control, column B: DHEA 10 μM, column C: DHEA 50 μM, column D: DHEA 100 μM. Line 1: unstimulated cells, line 2: oncostatin M (2.5 ng/ml)-induced cells, line 3: bFGF (25 ng/ml)-induced cells, line 4: VEGF (20 ng/ml)-induced cells, (n=4). (B) Effect of DHEA on capillary tube formation, in a fibrin gel. Capillary tube formation was analysed in vehicle-treated control cells and cells treated with DHEA, for 3 days, with an inverted microscope. Panel A: control, panel B: DHEA 1nM, panel C: DHEA 10 nM, panel D: DHEA 100 nM, panel E: DHEA 1 μM, panel F: DHEA 10 μM, panel G: DHEA 50 μM, panel H: DHEA 100 μM (Scale bar=200 μm).

3.3. Effect of DHEA(S) on capillary tube formation, in a three-dimensional fibrin gel

After 3 days of culture in a fibrin matrix, the formation of capillary tube-like structures was observed under an inverted microscope. DHEAS and DHEA, at physiological concentrations, had no effect on the formation of capillary tubes-like structures in a fibrin matrix (Fig. 2B, panels: B, C, D, E). In contrast, DHEA at higher concentrations (10, 50 and 100 μM) considerably reduced the number and length of capillary tubes (Fig. 2B, panels F, G, H). This dose-dependent inhibitory effect on capillary tube formation was striking at 10 μM DHEA.

3.4. Influence on cytoskeleton organisation

Actin filaments were examined by confocal microscopy after TRITC-phalloidin labelling of HMEC-1 that had been incubated for 24 h with bFGF (25 ng/ml) in the absence or the presence of DHEA. Actin stress fibres were not significantly altered by DHEA, even at concentrations that inhibited endothelial cell migration (Fig. 3A).

The tubulin-microtubular network was analysed by indirect immunofluorescence microscopy. It appeared to radiate outwards from the perinuclear region to the plasma membrane in untreated HMEC-1cells. DHEA was ineffective at 10 μM, whereas at 50 and 100 μM it altered the cellular network of tubulin in HMEC-1, leading to tubulin condensation around the nucleus (Fig. 3B).

3.5. Effect of DHEA on G6PDH activity by endothelial cells

In unstimulated HMEC-1, DHEA had a mild inhibitory effect (29% decrease at 100 μM) on G6PDH activity. Treatment with bFGF slightly increased G6PDH activity in endothelial cells (115% of activity in bFGF-treated cells as compared to unstimulated cells). In bFGF-stimulated cells, the inhibition of G6PDH activity by

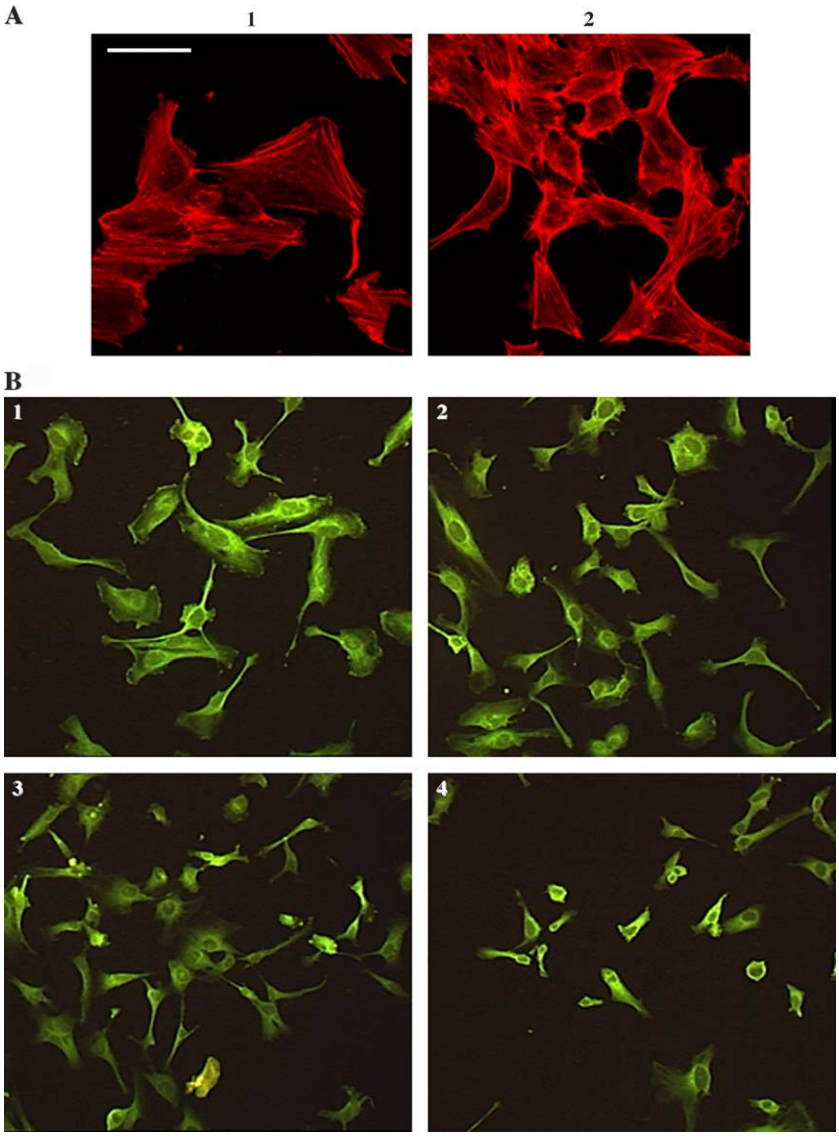

Fig. 3. Effect of DHEA on cytoskeleton organisation. (A) Effect of DHEA on actin stress fibres. The organisation of the actin cytoskeleton was analysed by confocal microscopy in bFGF-stimulated endothelial cells, after a 24-h incubation with DHEA. Panel 1: control, panel 2: DHEA 100 μM (Scale bar=10 μm). (B) Effect of DHEA on β-tubulin. The organisation of the tubulin network was analysed by immunofluorescent microscopy on bFGF-stimulated endothelial cells, after a 48-h incubation with DHEA. Panel 1: control, panel 2: DHEA 10 μM, panel 3: DHEA 50 μM, panel 4: DHEA 100 μM (magnification ×50).

DHEA was stronger than that in unstimulated cells, as the activity decreased from 115% to 54%.

3.6. Metabolism of ^{14}C-DHEA by endothelial cells in vitro

Radioactive metabolites formed after the incubations of HMEC-1 with ^{14}C-DHEA were characterised by one-dimensional TLC, scanning and autoradiography. Only two radioactive areas, other than that of the substrate, were located on radiochromatograms. One radioactive spot had an Rf value corresponding to authentic ADIOL. The other was more polar than ADIOL and could not be characterised because of the lack of a reference steroid. About 5–10% of ^{14}C-DHEA was converted into ADIOL and about 2–6% into the polar metabolite, in both unstimulated and bFGF-stimulated HMEC-1, after a 24-h incubation period (Table 1). In control incubations, with culture medium alone, the background radioactivity at the position of ADIOL and the polar metabolite did not exceed 0.2% of incubated radioactivity.

3.7. Influence of high concentrations of DHEA on angiogenesis in vivo

In the absence of bFGF, only a few vessels were observed in the mouse Matrigel plug, and the presence of DHEA did not modify the angiogenic index (Fig. 4). As expected, the presence of bFGF significantly induced the formation of neovessels in vivo. In DHEA-enriched Matrigel, the bFGF-

Table 1
Metabolism of ^{14}C-DHEA by endothelial cells, in vitro

Metabolic conversion rates (pmol/10^6 cells/24 h)		
	Unstimulated HMEC-1	bFGF-stimulated HMEC-1
ADIOL	9.1±0.7	6.4±0.8
Polar metabolite	5.6±0.2	3.8±1.0

HMEC-1 were incubated in the presence or absence of bFGF (25 ng/ml) with 100 nM ^{14}C-DHEA for 24 h. Media were collected and cells resuspended in PBS and counted. Extracted steroids from media were submitted to thin-layer chromatography (TLC). Quantitation of radiometabolites was performed by the Multitrace Master. Rates of metabolic conversion are expressed in pmol of product formed/10^6 cells/24 h (mean±S.E.M.; n=3).

induced formation of new blood vessels was strongly decreased (70% inhibition, Fig. 4).

4. Discussion

DHEA supplementation is commonly used in the elderly, in the hope of preventing or treating deleterious effects associated with ageing. In addition, angiogenesis-dependent diseases, such as cancer, atherosclerosis and macular degeneration, are frequent in the elderly and some steroids, including estrogens, have previously been shown to be either anti-angiogenic or angiogenic in vitro and in vivo (Banerjee et al., 1997; Folkman and Ingber, 1987; Fotsis et al., 1994; Lansink et al., 1998; Ma et al., 2001). For these reasons, we evaluated the actions of DHEA(S) on angiogenesis, using a wide range of steroid concentrations in order to test those reached in plasma after oral administration of DHEA (50 mg/day) and those reached in tissue after local administration or storage (Casson et al., 1996; Feher and Bodrogi, 1982; Feher et al., 1976).

For in vitro studies, capillary endothelial cells, HMEC-1, were used because they are representative of microvascular endothelial cells and because angiogenesis occurs in the microvasculature but not in large blood vessels (Klagsbrun and Folkman, 1990). In a set of experiments, the action of DHEA was evaluated in the presence of different angiogenic factors VEGF or bFGF, which are mostly involved in tumour angiogenesis, or oncostatin M, which is implicated in atherosclerotic plaque angiogenesis (Vasse et al., 1999). We showed that low concentrations were devoid of angiogenic activity, while higher concentrations, from 50 μM, inhibited capillary tube formation in a tridimensional gel structure. This inhibition was associated with a decrease in endothelial cell proliferation related to a blockage in G2/M phase (dose-dependant inhibition between 10 and 100 μM DHEA) and was more pronounced when endothelial cells were stimulated with angiogenic factors. It was also associated with a decrease in endothelial cell migration, in a wound-healing assay, under conditions in which "wound" repair was mostly due to cell migration rather than cell proliferation because of very low concentrations of fetal calf serum in the culture medium. This inhibition of cell migration was not associated with a decreased secretion of MMP-2 or MMP-9, as the pattern of gelatin degradation was unchanged in the presence of DHEA (results not shown). Interestingly, this inhibition of angiogenesis by high concentrations of DHEA was confirmed in vivo, as shown by the decrease in the angiogenic index in bFGF-enriched Matrigel plugs, in the presence of DHEA, in Nude mice.

The anti-angiogenic activity of high micromolar concentrations of DHEA may involve several mechanisms: the one that is more likely to be responsible for the inhibitory effect of DHEA on angiogenesis is the cytoskeleton organisation, which is known to play a crucial role in both cell proliferation and locomotion (Jordan and Wilson, 1998). While DHEA did not affect the pattern of actin fibres in HMEC-1, it clearly modified the tubulin distribution. The involvement of the disorganisation of the tubulin network in the inhibitory action of high concentrations of DHEA on angiogenesis is consistent with a modification of cell migration and mitosis (Mounetou et al., 2001). In addition, the decrease in G6PDH activity evoked by DHEA in endothelial cells may also participate in this inhibition of angiogenesis. Indeed, DHEA has already been shown to be a non-competitive inhibitor of G6PDH (Gordon et al., 1995), and G6PDH activity is important for cell proliferation since it participates in the formation of nucleotides through the synthesis of ribose-5-phosphate (Tian et al., 1998). In addition, it has recently been reported that G6PDH activity is involved in VEGF-mediated angiogenesis (Leopold et al., 2003). However, this mechanism seems to be of less importance than the tubulin disorganisation, because the cell cycle was inhibited in G2/M.

The actions of DHEA on angiogenesis may involve the steroid per se or one of its metabolites produced in endothelial cells. Although the potential metabolic transformation of DHEA into DHEAS has not been examined in HMEC-1, the present data indicated that DHEAS, even at high concentrations, did not affect either endothelial cell proliferation or

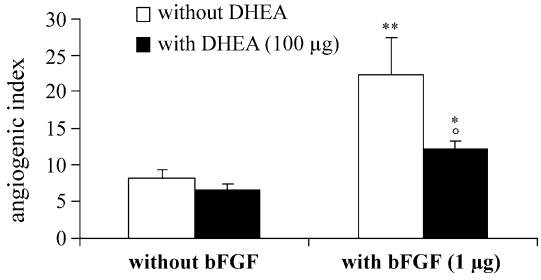

Fig. 4. Effect of DHEA on angiogenesis in vivo, in the Matrigel plug assay, in nude mice. Matrigel (300 μl) mixed with or without bFGF (1 μg) and with vehicle alone or with DHEA (100 μg) was subcutaneously injected into nude mice. Each group contained five mice. After 10 days, plugs were removed and paraffin-embedded. Sections from different conditioned Matrigel plugs were submitted to immunohistological analysis, using an antibody against mouse platelet-endothelial cell adhesion molecule 1. Angiogenesis was quantified according to Weidner's method. Results are expressed as the mean angiogenic index of each group ±S.E.M. *P<0.05 and **P<0.01 compared with the group without bFGF and without DHEA, °P<0.05 compared with the bFGF-group.

migration, or capillary tube formation in a fibrin gel. The inability of DHEAS to act on endothelial cells, in vitro, may be due to the lack of a specific transporter for this anionic steroid conjugate across the endothelial cell plasma membrane (Gordon et al., 1995; Kullak-Ublick et al., 1998). In addition, we showed that endothelial cells were able to convert, in low amounts, ^{14}C-DHEA mainly into its C17-reduced derivative, ADIOL. The biosynthesis of ADIOL is consistent with the presence of 17β-hydroxysteroid-oxydoreductase activity in endothelial cells, as also reported previously in classical steroidogenic tissues, such as placenta, ovaries and testis (Labrie et al., 2001), and in the aged human brain (Weill-Engerer et al., 2003). The formation of a very polar metabolite was also detected by thin-layer chromatography analysis. This steroid might correspond to the 7α-hydroxylated derivative of ADIOL (although this very polar metabolite could not be identified because of the lack of steroid standards), and data are consistent with the presence of 7α-hydroxylase activity, as previously demonstrated in several tissues (Akwa et al., 1992; Labrie et al., 2001; Weill-Engerer et al., 2003). The Rf of this very polar steroid (Akwa et al., 1992, 1993) means that the formation of 7α-hydroxy-DHEA can be excluded. Furthermore, under our experimental conditions, ^{14}C-DHEA was not metabolised into Δ4-androstenedione, testosterone or 17β-estradiol because no metabolite other than the two above-mentioned were observed. The potential effect of ADIOL on angiogenesis awaits further investigation.

In conclusion, the present study demonstrated that DHEA, at pharmacological concentrations, which could be reached when it is applied locally or stored in a tissue, inhibited angiogenesis both in vitro and in vivo. This effect can be attributed to DHEA per se or possibly to its metabolite ADIOL, which is synthesised in HMEC-1. In contrast, DHEA at physiological concentrations and DHEAS at any concentration used were totally devoid of activity on angiogenesis. However, this study cannot exclude an indirect deleterious effect on angiogenesis by DHEA via its metabolites formed in macrophages or others cells (Labrie et al., 2001; Schmidt et al., 2000) or by the stimulation of angiogenic factor secretion in vivo. Further investigations are required to ascertain the potential beneficial role of high concentrations of DHEA on the inhibition of cell proliferation and dissemination in angiogenesis-dependent tumours.

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