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Differential effects of angiostatic steroids and dexamethasone on angiogenesis and cytokine levels in rat sponge implants

Yozo Hori, De-En Hu, †Kiyoshi Yasui, Rachel L. Smither, *G. Austin Gresham & [1]Tai-Ping D. Fan

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ; *Department of Histopathology, Addenbrooke's Hospital, Cambridge CB2 2QQ & †Shionogi Research Laboratories, Shionogi & Co., Ltd., 3-1-1 Futaba-cho, Toyonaka, Osaka 561, Japan

1 Subcutaneous implantation of sterile polyether sponges elicited a reproducible neovascular response in rats, as determined by blood flow measurement with a ^{133}Xe clearance technique and confirmed histologically. This model was used to monitor the levels of two cytokines during angiogenesis and to compare the activities of angiostatic steroids and anti-inflammatory steroids.

2 Initial experiments followed the neovascular development over a 20-day period. Daily local injection of hydrocortisone caused a dose-dependent (0.5, 5 and 50 μg per sponge) inhibition of the basal sponge-induced angiogenesis. However, daily systemic treatment of hydrocortisone (2, 10 and 50 mg kg^{-1}, s.c.) was less effective at inhibiting angiogenesis, and this inhibition was not sustained by day 20 after sponge implantation.

3 To investigate the involvement of cytokines during the course of angiogenesis, we measured the endogenous levels of tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) in sponge implants. Levels of IL-6 and TNF-α peaked at day 7 and day 11 after implantation, respectively. These cytokine levels subsided through the completion of angiogenesis by day 20.

4 Subsequent experiments were carried out over a 14-day period. Among the three angiostatic steroids tested, U-24067 (6α-fluoro-17,21-dihydroxy-16α-methylpregna-4,9(11)-diene-3,20-dione-21-acetate) showed a dose-dependent inhibition (0.5, 5 and 50 μg per sponge per day) of sponge-induced angiogenesis. Tetrahydro-S was also effective at 5 μg doses, but medroxyprogesterone failed to affect the angiogenic response. None of these steroids caused atrophies of the spleen and thymus.

5 Daily local injection of dexamethasone (0.5 μg per sponge) inhibited the basal sponge-induced angiogenesis almost completely. Although higher doses of dexamethasone (5 and 50 μg per sponge) did not produce further inhibition of angiogenesis, they caused severe spleen and thymus weight losses, indicative of immunosuppression.

6 At the daily dose of 5 μg per sponge, dexamethasone inhibited angiogenesis and produced a marked reduction in the levels of TNF-α and IL-6 at day 14. In contrast, hydrocortisone, U-24067 and tetrahydro-S did not influence the levels of TNF-α and IL-6.

7 We concluded that the anti-angiogenic activity of angiostatic steroids and anti-inflammatory steroids in the rat sponge model is independent of their ability to reduce the production of TNF-α and IL-6. The differential effects of angiostatic and anti-inflammatory steroids suggest that U-24067 and its derivatives may have therapeutic potential in the management of angiogenic diseases such as rheumatoid arthritis.

Keywords: Angiogenesis; angiostatic steroids; anti-inflammatory steroids; tumour necrosis factor-α; interleukin-6; inflammation

Introduction

Angiogenesis can be defined as the development of new blood vessels from an existing vascular bed. Normal vascular proliferation occurs only during embryonic development, the female reproductive cycle and wound repair. By contrast, many pathological conditions (e.g. cancer, rheumatoid arthritis, atherosclerosis and diabetic retinopathy), are characterized by persistent, unregulated angiogenesis. Thus, an anti-angiogenic strategy may prove a novel therapeutic approach for the treatment of these diseases (Folkman & Shing, 1992; Colville-Nash & Scott, 1992; Peacock *et al.*, 1992; Folkman, 1995). Since the first successful clinical treatment of pulmonary haemangioendotheliomas with interferon α-2a, several anti-angiogenic agents are currently being evaluated for their efficacy as anti-cancer drugs (reviewed in Fan *et al.*, 1995).

In 1983, Folkman *et al.* showed that the combination of heparin or a heparin fragment with cortisone was able to inhibit angiogenesis and tumour growth. Subsequent work led to the discovery of a new class of steroids that inhibits angiogenesis in the chick chorioallantoic membrane (CAM) in the presence of non-anticoagulant heparin (Crum *et al.*, 1985). Since the anti-angiogenic activity of these steroids is independent of their glucocorticoid activity, they are designated as angiostatic steroids. However, the requirement of heparin for the activity of these new steroids has been much debated. For example, Lee *et al.* (1990) and Tamargo *et al.* (1990) found that the combination of heparin and hydrocortisone or cortisone significantly suppressed the angiogenic response and tumour growth. In a cotton pellet granulomatous inflammation model, cortisone/heparin combination was also shown to retard the growth of granulomatous tissue, mononuclear cell infiltration and cartilage degradation (Colville-Nash *et al.*, 1993). In contrast, several groups failed to observe the synergistic effect of heparin and angiostatic steroids especially in tumour-induced angiogenesis (Penhaligon & Camplejohn, 1985; Ziche *et al.*, 1985; Teale *et al.*, 1987). The discrepancy of these results may be due to the heterogeneity of heparin preparations (Folkman *et al.*, 1989; Seed & Colville-Nash, personal communication), or the differences in degradation of steroids or of heparin by different types of tumours (Folkman & Brem, 1992). Alternatively, angiogenesis in mast-cell-rich inflammatory lesions or haemangiomas can sometimes be blocked by corticosteroids alone, due to the high levels of endogenous heparin (Folkman & Brem, 1992).

[1] Author for correspondence.

Glucocorticoids are commonly used in the treatment of chronic inflammatory diseases such as rheumatoid arthritis. In addition to their anti-inflammatory and immunosuppressive actions, these steroids are also anti-angiogenic (Folkman & Ingber, 1987). In view of the paradoxical effects of heparin outlined above, we decided to carry out a comparative study of glucocorticoids and angiostatic steroids (Crum *et al.*, 1985; Wilks *et al.*, 1991) in its absence in the rat sponge implant model (Andrade *et al.*, 1987; Hu *et al.*, 1995). Because the sponge wound fluids can be extracted easily for analysing the profiles of cytokines, we also investigated the relative potency of these steroids in modifying the levels of two angiogenic peptides, tumour necrosis factor-α (TNF-α; Leibovich *et al.*, 1987; Fràter-Schröder *et al.*, 1987; Fajardo *et al.*, 1992) and interleukin 6 (IL-6; May *et al.*, 1989; Motro *et al.*, 1990).

Methods

The rat sponge model

Circular sponge discs (5 mm thick, 1.2 cm diameter) were prepared from a sheet of polyether polyurethane foam by use of a wad punch. A 1.2 cm segment of polythene tubing (1.4 mm internal diameter; Portex Ltd., Hythe) was secured to the interior of each sponge disc by means of 5/0 silk sutures so that every sponge disc had a central cannula. Sponge discs were soaked in 70% ethanol for 2–3 h and then rinsed in sterile PBS without calcium and magnesium. After the sponges had been squeezed in a 20 ml syringe to remove the excess PBS, they were exposed to ultraviolet light overnight.

Male Wistar rats (180–200 g) were anaesthetized with Hypnorm (0.5 ml kg^{-1}, i.m.) and sponge discs with attached cannulae were implanted subcutaneously as described previously (Andrade *et al.*, 1987; Hu *et al.*, 1995). To prevent the rats from tampering with the cannulae, they were housed individually in plastic cages with free access to food and water.

Neovascularisation was assessed as a function of blood flow through the implants over a period of 14 or 20 days, by a ^{133}Xe clearance technique (Andrade *et al.*, 1987). Briefly, animals were anaesthetized with Hypnorm as before and 10 μl ^{133}Xe in sterile PBS was injected into the sponges through the cannulae. The washout of radioactivity from the implants was monitored by a gamma scintillation detector and the 6 min ^{133}Xe clearance value was calculated as a percentage of the initial count. The validity of this method has recently been established (Hu *et al.*, 1995).

Test compounds were administered subcutaneously (2 ml kg^{-1} daily) or into sponges through attached cannulae (50 μl per sponge daily), starting on day 1 after implantation. To exclude the possible acute effects of the test compounds (dilatation or constriction) on the microvasculature, they were given 16–24 h before the ^{133}Xe measurements.

At the end of experiments, the animals were killed by cervical dislocation and their body weights recorded. The sponges, spleen and thymus from each animal were dissected out carefully and weighed.

Histology

The sponges were bisected and fixed in formal saline at 4°C for 1 h and then immersed in 75% ethanol for 30 min and finally kept in 90% ethanol. Paraffin sections (10 μm) were prepared and stained with haematoxylin and eosin. Alternatively, blood vessels were visualized with a specific endothelial cell marker $BSL\text{-}B_4$ (Laitinen, 1987) by use of an immunoperoxidase technique. Each slide was subjected to standard H_2O_2 and trypsin treatments and then incubated with 0.5 ml of $BSL\text{-}B_4$ (1:5 dilution in tris buffered saline (TBS), pH 7.6) at room temperature for 60 min. Following three 10-min washes in TBS, the slide was developed with 0.5 ml of streptavidin-biotin complex for 30 min, washed in TBS again and finally counterstained with Harris haematoxylin.

Cytokine assays

Sample preparation Sponge implants were collected at fixed time intervals after implantation. After being weighed, they were homogenised in 2 ml of ice-cold PBS with Polytron for 30 s. The homogenates were then centrifuged at 2,000 *g* for 30 min, and the supernatants dialysed with PBS for 24 h, and for another 24 h with RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan). Samples were sterilised with Millipore filters (pore size, 0.45 μm) and stored at −70°C until assay. Protein content in samples was determined by use of the BCA Protein Assay Reagent (Pierce Chemical Ltd., Rockford, IL, U.S.A.).

Cytokines and cell lines Recombinant murine TNF-α (Genzyme, Cambridge, Mass., U.S.A.) has 4×10^7 units mg^{-1} as determined by L929 assay. L-M (L-929 subclone) murine fibroblast cell line for assay of TNF-α was a kind gift from Dr K. Uno (Institut Pasteur de Kyoto, Kyoto, Japan). Recombinant human IL-6 was a kind gift from Dr T. Hirano (Biomedical Research Center, Osaka University Medical School, Osaka, Japan) and has 5.3×10^6 units mg^{-1} as determined by an IL-6-dependent murine hybridoma clone, MH60.BSF2 (Matsuda *et al.*, 1988), which was also kindly provided by Dr T. Hirano.

TNF-α bioassay

TNF-α levels were assayed by its cytotoxicity for L-M cells as described by Aggawal *et al.* (1985). Briefly, 5×10^4 cells were cultured in a final volume of 200 μl with 2 fold dilution of samples or standards in Eagles minimum essential medium (MEM, Irvine Scientific Co., U.S.A.) containing 1% FBS, non-essential amino acids for MEM, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin and 1 μg ml^{-1} actinomycin D. After 20 h incubation, cell number was evaluated by a modified colorimetric MTT assay (Mosmann, 1983). Twenty five microlitres of MTT (6 mg ml^{-1}) was added to the wells and the plates were incubated at 37°C for a further 4 h. This was followed by the addition of 50 μl lysing buffer (20% sodium dodecyl sulphate in 0.02 M HCl). The plates were then left overnight at 37°C and the optical densities were read at 540 nm with a Titertek plate reader. TNF-α activity was expressed as equivalents of standard recombinant murine TNF-α.

IL-6 bioassay

Levels of IL-6 were measured by use of an IL-6-dependent murine hybridoma clone, MH60.BSF2, as described previously (Matsuda *et al.*, 1988). Briefly, 1×10^4 cells were cultured in a final volume of 200 μl with 2 fold dilution of samples or standards in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 u ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin. After 44 h incubation at 37°C in an atmosphere of 5% CO_2 and 95% air, cell proliferation was evaluated by a modified colorimetric MTT assay as described above. IL-6 activity in samples was expressed as equivalents of standard recombinant human IL-6.

Chemicals and other materials

Hypnorm (0.315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone) was from Janssen Pharmaceuticals (Oxford). Dexamethasone-21-phosphate disodium, hydrocortisone-21-phosphate disodium, medroxyprogesterone acetate (6α-methyl-17α-hydroxyprogesterone acetate), tetrahydro-S (5β-pregnane-3α,17α,21-triol-20-one), bovine serum albumin (BSA) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were all from Sigma Chemical Co. Ltd. (Poole). U-24067 (6α-fluoro-17,21-dihydroxy-16α-me-

thylpregna-4,9(11)-diene-3,20-dione-21-acetate) was a generous gift from Dr J. W. Wilks (The Upjohn Company, Kalamazoo, MI, U.S.A.). ^{133}Xe injection (370 MBq in 3 ml saline) was obtained from Amersham International plc (Little Chalfont). All other chemicals used were of reagent grade or of the purest commercially available grade unless otherwise stated. Polyether polyurethane foam was purchased from R E Carpenter & Co. (High Wycombe). *Bandeiraea simplicifolia* lectin I, isolectin B_4 ($BSL-B_4$; No. B1205) and VECTASTAIN ABC kit were from Vector Laboratories (Peterborough).

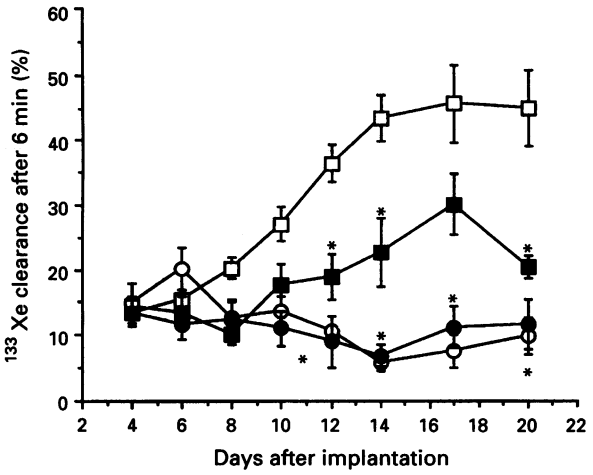

Figure 1 Effect of hydrocortisone on sponge-induced angiogenesis. Hydrocortisone at 0.5 (■), 5 (○) and 50 μg (●) per sponge or phosphate buffered saline (PBS, □) was administered once daily as a 50 μl bolus into the sponges from day 1 to day 19. Data represent the mean ± s.e.mean, $n = 8-10$. *$P < 0.05$ (5, 50 μg hydrocortisone vs PBS on days 12, 14, 17 and 20; 0.5 μg hydrocortisone vs PBS on days 12, 14 and 20).

Dexamethasone and hydrocortisone were prepared in phosphate buffered saline (PBS, Dulbecco's ICN Biomedicals Ltd., Thame). Medroxyprogesterone and tetrahydro-S were prepared as a stock solution of 20 mg ml^{-1} in ethanol and diluted with PBS containing 0.1% BSA. U-24067 was solubilized to 33 mg ml^{-1} in acetone:methanol (1:1, v/v) and diluted with PBS containing 0.1% BSA. All drug solutions were filter (0.22 μm) sterilised (ICN Biomedicals Ltd., Thame) before use.

Statistics

Statistical analysis was done by StatView II (Abacus Concepts, Berkeley, CA, U.S.A.) on a Macintosh computer. As appropriate, statistical significance was tested by Student's *t* test, or, for multiple comparisons, one-way analysis of variance, followed by Fisher PLSD test.

Results

Effect of hydrocortisone on ^{133}Xe clearance from vascularised sponges

Initial experiments followed the neovascular development over a 20-day period. Figure 1 shows that in control sponges the ^{133}Xe clearance was between 13% and 15% during the first 6 days after implantation due to passive diffusion of the ^{133}Xe from the sponges. After day 6 the clearance increased gradually so that by day 14 it was 45%. This level of ^{133}Xe clearance was maintained up to day 20.

Daily local injection of 0.5, 5 and 50 μg hydrocortisone caused a dose-dependent reduction of ^{133}Xe clearance from the sponges over the 20-day period (Figure 1), although the highest doses of hydrocortisone (50 μg per sponge) did not produce additional inhibition over that achieved at 5 μg. However, systemic treatment of hydrocortisone (2, 10 and

Table 1 Effects of glucocorticoids and angiostatic steroids on ^{133}Xe clearance from sponge implants and the weights of spleen and thymus at day 14

Drug	Daily dose	^{133}Xe cleared within 6 min (%)	Weight (mg) Spleen	Thymus
Hydrocortisone	Vehicle	41.3±7.7	650±27	320±26
	0.5 μg per sponge	22.8±5.3	597±38	295±2
	5	5.9±1.3*	712±47	360±15
	50	6.9±1.7*	723±45	328±44
Hydrocortisone	Vehicle	42.8±5.1	723±40	343±36
	2 mg kg^{-1}, s.c.	42.7±4.7	702±52	242±17*
	10	32.8±5.8	655±61	196±3*
	50	21.8±3.5*	542±24	91±7*
Dexamethasone	Vehicle	44.9±2.5	835±61	304±28
	0.5 μg per sponge	7.8±0.8*	660±18*	206±37
	5	15.1±3.4*	538±46*	64±10*
	50	14.1±3.5*	251±18*	36±4*
U-24067	Vehicle	42.1±5.6	665±29	391±10
	0.5 μg per sponge	30.2±4.7	732±43	394±13
	5	24.2±4.2*	720±42	431±26
	50	20.8±4.1*	766±5*	455±42
Tetrahydro-S	Vehicle	40.5±3.5	723±15	312±20
	0.5 μg per sponge	42.7±5.2	711±55	364±26
	5	25.4±2.5*	679±42	301±28
	50	32.7±4.7	720±72	294±27
Medroxyprogesterone	Vehicle	42.4±3.4	672±42	337±24
	0.5 μg per sponge	32.4±6.3	683±19	364±27
	5	36.3±8.1	738±44	322±34
	50	31.6±7.4	713±81	359±53

Drugs were administered daily from day 1 to day 13. Values are mean ± s.e.mean, $n = 8-10$ for ^{133}Xe clearance and $n = 4-5$ for the spleen and thymus weight. *$P < 0.05$ vs vehicle control (Fisher PLSD test following one-way ANOVA).

50 mg kg^{-1}, s.c.) was less effective in inhibiting angiogenesis (Table 1), and this inhibition was not sustained by day 20 after sponge implantation (data not shown).

Effects of angiostatic and anti-inflammatory steroids on ^{133}Xe clearance from sponges and spleen and thymus weights

In subsequent experiments, a 14-day time course was followed. Figure 2a shows that the angiostatic steroid U-24067 produced a dose-dependent inhibition of ^{133}Xe clearance from the sponges. The anti-angiogenic action of daily doses of 5 and 50 μg was sustainable for the 14-day period, while daily doses of 0.5 μg could only suppress ^{133}Xe clearance up to 10 days. In contrast, daily local injection of 0.5 μg of the anti-inflammatory steroid dexamethasone produced almost complete inhibition of ^{133}Xe clearance from the sponges over the entire 14-day period (Figure 2b). Higher doses of dexamethasone (5 and 50 μg per sponge) did not appear to produce any additional inhibition.

Table 1 summarizes the effects of the different steroids on the ^{133}Xe clearance from sponge implants and the weights of the spleen and thymus of the animals. Among the three angiostatic steroids tested, only U-24067 (0.5, 5, 50 μg per sponge daily) showed dose-dependent inhibition of ^{133}Xe clearance. Tetrahydro-S was effective only at 5 μg per sponge daily, while medroxyprogesterone failed to affect ^{133}Xe clearance.

Of all the compounds tested, only subcutaneous administration of hydrocortisone and local injection of dexamethasone caused a profound and dose-dependent reduction in the weights of spleen and thymus (Table 1).

Histology

The inhibitory effect of the steroids on the sponge-induced angiogenesis was confirmed histologically. These sponges had been treated with PBS or different steroids for 13 days and collected at day 14. Figure 3a clearly shows that control sponge implants were encapsulated with collagenous connective tissue consisting mainly of fibroblasts. Capillaries and existing arterioles were evident in vascularized regions which were also diffusely infiltrated by lymphoid cells. *Bandeiraea simplicifolia* lectin I, isolectin B_4 proved to be an excellent endothelial cell marker, revealing numerous canalised capillaries and non-canalised angiogenic buds (Figure 3b). At a daily dose of 5 μg, dexamethasone profoundly inhibited cellular infiltration (Figure 3c) and capillary formation leaving fibrinous strands and cell debris in the sponge matrix (Figure 3d). Similar results were obtained with 5 μg daily doses of U-24067 (Figure 3e), hydrocortisone and tetrahydro-S (data not shown). In contrast, medroxyprogesterone failed to affect the sponge-induced angiogenic response. The fibroblastic capsule was clearly evident; plasma cells and other mononuclear cell infiltrate were found between the capsule and areas of neovascularization within the sponge matrix (Figure 3f).

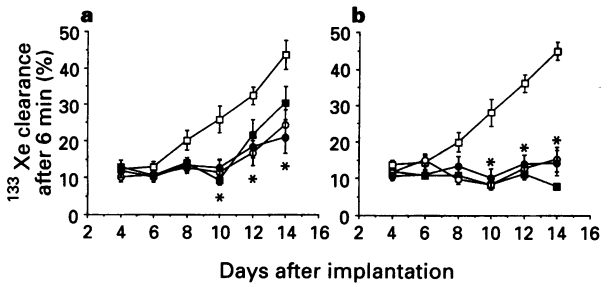

Figure 2 Effect of U-24067 and dexamethasone on sponge-induced angiogenesis. (a) U-24067 at 0.5 (■), 5 (○) and 50 μg (●) per sponge or phosphate buffered saline (PBS, □) was administered once daily as a 50 μl bolus into the sponges from day 1 to day 13. *$P<0.05$ (0.5 μg vs PBS on day 10 only; 5 and 50 μg vs PBS from days 10 to 14. (b) Dexamethasone at 0.5 (■), 5 (○) and 50 μg (●) per sponge or PBS (□) was administered once daily as a 50 μl bolus into the sponges from day 1 to day 13. *$P<0.05$ (0.5, 5, 50 μg dexamethasone vs PBS from days 10 to 14). Data represent the mean ± s.e.mean, $n=8-10$.

Profiles of TNF-α and IL-6 levels in sponge implants

In order to measure cytokine levels in sponge implants, another group of rats was implanted with sponges and injected locally with PBS (50 μl per sponge daily). The wet weights and protein contents of sponge implants peaked between day 7 and day 9, and gradually decreased thereafter (Figure 4a). During the course of angiogenesis, IL-6 levels reached a peak at day 7 and by day 20 it declined to a level similar to that of day 2 (Figure 4b). Levels of TNF-α showed a clear peak at day 11 and gradually decreased thereafter (Figure 4b).

Effects of steroids on TNF-α and IL-6 levels

Table 1 shows that all the steroids, except medroxyprogesterone, were able to inhibit ^{133}Xe clearance from vascularised sponges at a daily dose of 5 μg. Thus, this dose of the different steroids was chosen to compare their effects on the endogenous levels of TNF-α and IL-6 in sponge wound fluids at day 14. Table 2 shows that only dexamethasone significantly reduced the levels of IL-6 ($P<0.05$) and TNF-α ($P<0.05$). All other steroids produced no apparent effects on the levels of these two cytokines.

Discussion

Angiogenesis is a multi-step process which includes basement membrane degradation by metalloproteinases, endothelial cell migration, endothelial cell proliferation and basement membrane synthesis by endothelial cells of the newly formed capillaries. It also involves extensive interplay between cells, soluble factors and extracellular matrix components. Anti-angiogenic agents may be divided into two groups: (i) 'specific anti-angiogenic agents' – those that directly inhibit the growth of capillaries without affecting other tissues, and (ii) 'non-specific anti-angiogenic agents' – those that suppress neovascularisation indirectly via the inhibition of angiogenic influences/factors from cell types other than endothelial cells (Fan *et al.*, 1995). The angiostatic steroids are a novel group of compounds which do not have glucocorticoid activity (Crum *et al.*, 1985), but possess direct anti-angiogenic activity on vascular endothelial cells (see below; Folkman & Ingber, 1987; Sakamoto *et al.*, 1987; Stokes *et al.*, 1990). In contrast, dexamethasone is a potent anti-inflammatory/immunosuppressive glucocorticoid with a multiplicity of actions on a wide range of inflammatory/immune cell functions (Haynes, 1990). Here we have shown that both angiostatic steroids (U-24067, tetrahydro-S) and anti-inflammatory steroids (dexamethasone, hydrocortisone) inhibited angiogenesis as well as granuloma formation. To better understand the mechanism of actions of these two classes of steroids, we investigated their modulation of cytokines and influences on the reticuloendothelial system.

We have previously shown that the sponge-induced angiogenesis can be suppressed by a cocktail of IL-1 receptor antagonist and antibodies to IL-8, TNF-α and basic fibroblast growth factor (bFGF), suggesting that these cytokines may play an intrinsic role in angiogenesis (Hu *et al.*, 1994). In the present study, analyses of the profiles of TNF-α and IL-6 in the sponges revealed different kinetics of cytokine production: levels of IL-6 peaked on day 7, and TNF-α on day 11 after implantation. These cytokine levels subsided through the completion of angiogenesis as observed on day 20. TNF-α is a major cytokine produced by activated macrophages which has been shown to stimulate chemotaxis of capillary endothelial cells and to induce these cells to form tube-like structures in collagen gel (Leibovich *et al.*, 1987). Paradoxically, both IL-6

and TNF-α have been shown to inhibit the proliferation of vascular endothelial cells *in vitro* (May *et al.*, 1989; Fràter-Schröder *et al.*, 1987). IL-6 may exert its angiogenic activity indirectly by recruiting inflammatory cells into sponges to produce direct-acting angiogenic factors such as bFGF (Folkman & Shing, 1992).

Because all the steroids tested here, except medroxyprogesterone, were able to inhibit sponge-induced angiogenesis at a daily dose of 5 μg, this dose of the different steroids was chosen to compare their effects on the endogenous levels of TNF-α and IL-6 in sponge wound fluids. The results clearly showed that only dexamethasone reduced the levels of TNF-α and IL-6 in the sponges at day 14. True to their independence from glucocorticoid activity, the angiostatic steroids U-24067 and tetrahydro-S did not modify the levels of these cytokines. Taken together, these data exemplify the differential effects of anti-angiogenic and anti-inflammatory activity of these two distinct classes of steroids.

Glucocorticoids are known to be potent inhibitors of cytokine synthesis via the repression of cytokine gene transcription (Barnes & Adcock, 1993). For example, dexamethasone can inhibit the production of IL-6 from human monocytes, endothelial cells and fibroblasts (Waage *et al.*, 1990). It has also been shown to inhibit the production of IL-1 (Lee *et al.*,

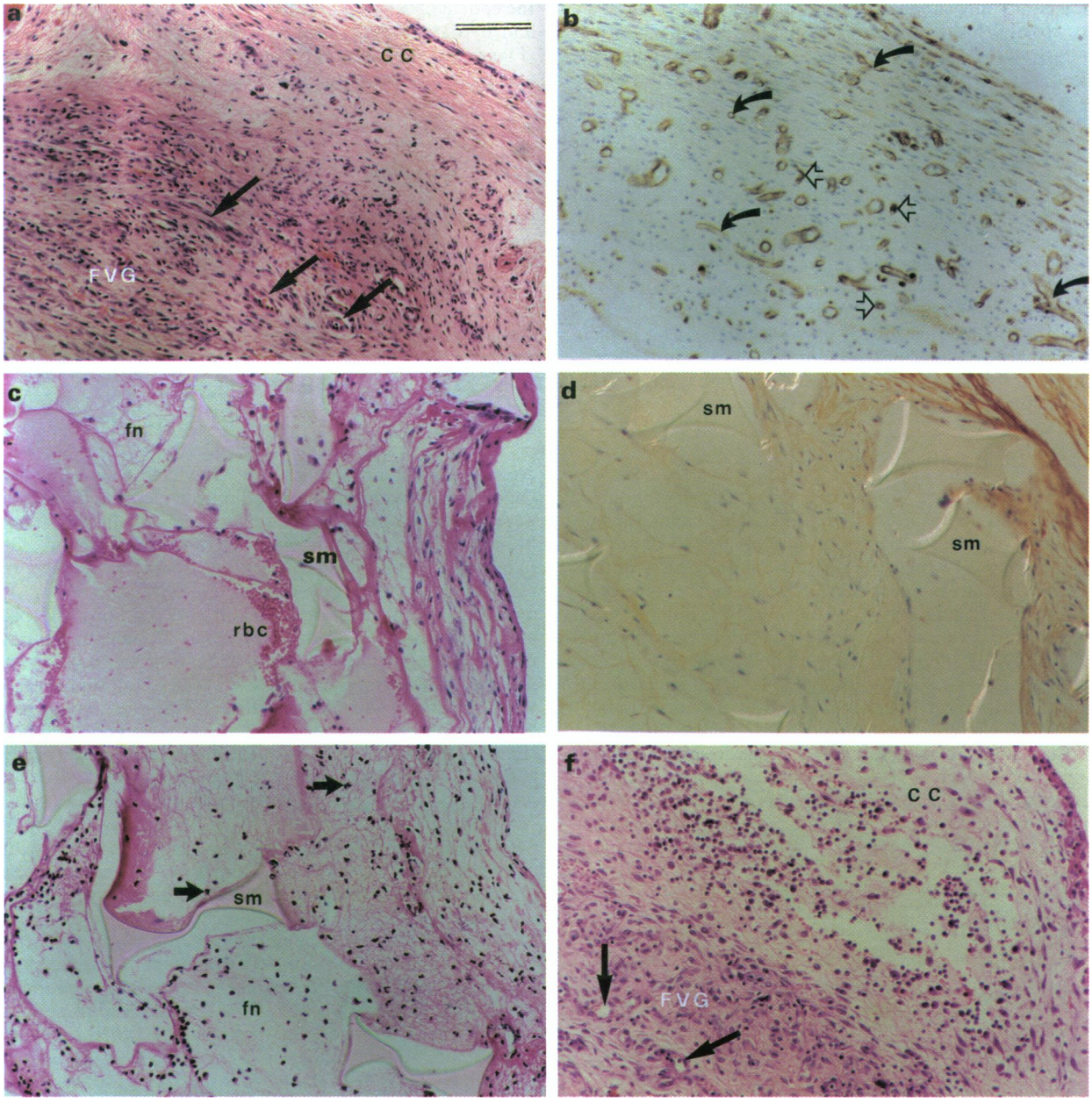

Figure 3 Histological section of 14-day-old sponges illustrating the effect of dexamethasone, U-24067 and medroxyprogesterone on cellular infiltration and neovascularization. All sections photographed at ×200 magnification. Bar = 100 μm. (a) Sponge treated with phosphate buffered saline (PBS, 50 μl; haematoxylin and eosin (H&E) stained), showing the well-organized collagenous capsule (cc), consisting of fibroblasts predominantly. The area of fibrovascular growth (FVG) had a good vascular network (large arrows), with neutrophils and lymphoid infiltration. (b) Similar sponge section as (a), stained with BSL-B_4 to reveal the extensive vascular network of canalized capillaries (curved arrows). Some of the labelled structures were probably non-canalized angiogenic buds (open arrows). (c) Sponge treated with dexamethasone (5 μg day^{-1}; H&E stained), showing a thin layer of fibroblasts encapsulating the sponge matrix (sm) which contained fibrinous exudate (fn) and red blood cells (rbc) only. (d) Similar sponge section as (c), stained with BSL-B_4 to confirm the total absence of angiogenesis. (e) Sponge treated with U-24067 (5 μg day^{-1}; H&E stained), showing a lack of neovasculature. Clusters of neutrophils were still evident (arrows). (f) Sponge treated with medroxyprogesterone (5 μg day^{-1}; H&E stained), confirming that the drug failed to affect the sponge-induced angiogenic response. The fibroblastic capsule (cc) and blood vessels (arrows) were clearly evident. Many plasma cells and other lymphocytic infiltrate were found between the capsule and areas of fibrovascular growth (FVG).

1988) and bFGF (Gay & Winkles, 1991) by appropriate target cells. Since the angiostatic steroids effectively suppressed angiogenesis without modifying the levels of TNF-α and IL-6, it is clear that the anti-angiogenic activity of angiostatic steroids and anti-inflammatory steroids in the rat sponge model is independent of their ability to reduce the production of TNF-α and IL-6. It is conceivable that the inhibitory effect of dexamethasone on cytokine production could simply be due to the doses used. In the CAM assay, dexamethasone (plus heparin) produced a bell-shaped curve of antiangiogenic activity (Folkman & Ingber, 1987). Thus, its anti-angiogenic/glucocorticoid activities could be differentiated in the sponge model at lower doses.

Recent studies suggest that inflammation can sometimes be dissociated from angiogenesis (Folkman & Brem, 1992). For example, it is possible to induce angiogenesis in the cornea without causing inflammation. On the other hand, neovascularization does not occur in acute inflammation, because the endothelium can regenerate to replace injured or dying endothelial cells. However, angiogenesis is a major vascular response in chronic inflammation, with the neovasculature facilitating the entry of monocytes and other inflammatory cells to inflamed joints or granulomata. Because of its extensive repertoire of actions, demamethasone could inhibit endothelial cells directly and/or block the interactions between cells, soluble factors and extracellular matrix components. In view of the role of adhesion molecules in angiogenesis (Koch *et al.*, 1995), it is possible for dexamethasone to block their expression, leading to angiosuppression. Furthermore, down-regulation of adhesion molecules would be expected to diminish the recruitment of leucocytes, resulting in lower concentrations of TNF-α, IL-6 and perhaps other cytokines in the sponges. This interpretation is supported by the work of Cronstein *et al.* (1992), who showed that dexamethasone and cortisol attenuate the expression of E-selectin and intracellular adhesion molecule 1 (ICAM-1) by lipopolysaccharide-stimulated endothelial cells. In contrast, the angiostatic steroid tetrahydrocortisol, an inactive metabolite of cortisol, did not modify the expression of E-selectin or ICAM-1.

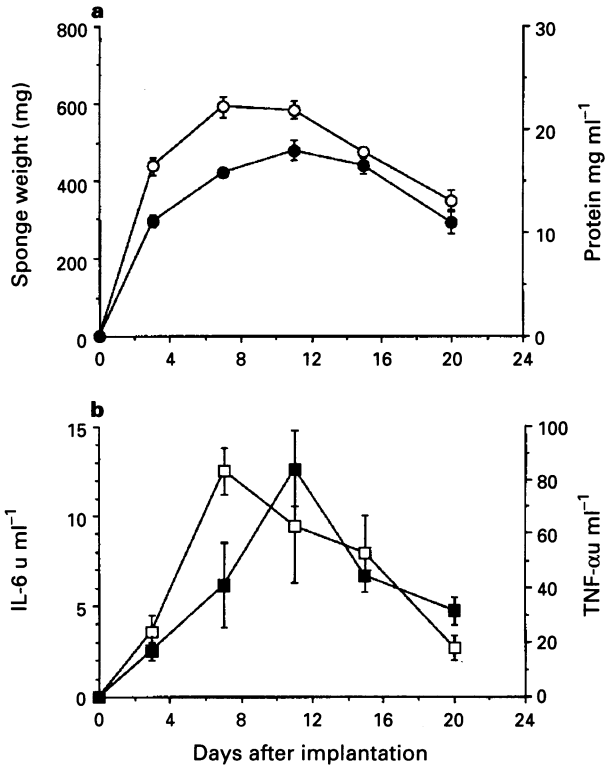

Figure 4 Time course in the changes of (a) sponge wet weight (○) and protein concentration (●), (b) tumour necrosis factor (TNF-α, ■) and IL-6 (□) levels in sponge implants. The sponges were treated with 50 μl day^{-1} of phosphte buffered saline. Data represent the mean ± s.e.mean, $n=4$.

In the present study, U-24067 and tetrahydro-S also caused an impressive reduction of leucocyte infiltration into the sponges. Because these agents do not possess glucocorticoid activity, as evidenced by their lack of effects on cytokine production and immune organ weights, one can argue that they inhibited leucocyte infiltration mainly as a result of their direct inhibition of neovascular growth. Similar results had been obtained for other angiogenic inhibitors. For example, AGM-1470 was able to suppress inflammatory infiltrate and neovascularization in a rat model of arthritis without producing immunosuppression (Peacock *et al.*, 1992). In a murine model of granuloma-mediated cartilage degradation, angiostatic steroid treatment decreased the vascularity of granulomatous tissue and retarded mononuclear cell infiltration (Colville-Nash *et al.*, 1993). Our data also confirm other findings that the angiostatic steroids can exert their actions without exogenous heparin. For example, U-24067 alone was able to inhibit bFGF- and sucralfate-induced angiogenesis in a novel CAM assay (Nguyen *et al.*, 1994). Subcutaneous injections of tetrahydrocortisol alone significantly inhibited angiogenesis in a murine chronic granulomatous air pouch model (Colville-Nash *et al.*, 1995).

How do angiostatic steroids inhibit angiogenesis? In theory, they can directly inhibit the growth of capillaries without affecting other tissues. Thus, the combination of β-cyclodextrin tetradecasulphate and hydrocortisone has been shown to inhibit migration of microvascular endothelial cells *in vitro* (Stokes *et al.*, 1990). Heparin and cortisone acetate inhibited endothelial cell growth both in cultures and in tumour masses (Sakamoto *et al.*, 1987). On the CAM, angiostatic steroid-heparin combinations induced basement membrane fragmen-

Table 2 Effects of glucocorticoids and angiostatic steroids on cytokine levels, sponge weight and protein content in sponge implants at day 14

Drugs (5 μg/sponge daily)	*IL-6* (u ml^{-1})	*TNF-α* (u ml^{-1})	*Sponge weight* (mg)	*Protein* (mg ml^{-1})
Vehicle	11.4±1.3	72.4±10.6	481±30	14.06±0.98
Hydrocortisone	16.7±8.6	72.6±9.8	515±28	13.86±0.88
Dexamethosone	5.1±1.3*	11.1±7.5*	480±23	12.99±1.07
Vehicle	9.4±3.4	81.5±17.6	453±17	14.48±0.87
Tetrahydro-S	14.9±2.4	79.6±7.7	474±18	16.57±0.76
Medroxyprogesterone	11.1±2.5	80.1±10.9	439±16	15.64±0.76
Vehicle	9.0±2.9	94.0±33.7	474±9	11.32±0.64
U-24067	4.0±1.2	84.8±28.6	426±21	10.56±0.42

Drugs (5 μg/sponge daily) were injected locally from day 1 to day 13. Values are means ± s.e.mean, $n=5-6$. $P<0.05$ vs vehicle control (Fisher PLSD test following one-way ANOVA). There is no significant difference between vehicle control and U-24067-treated group as determined by Student's *t*-test.

tation of growing capillaries (Ingber *et al.*, 1986) and switched growing capillaries into a regressive mode through alterations of collagen metabolism that result in the loss of extracellular matrix structural integrity (Ingber & Folkman, 1988; Maragoudakis *et al.*, 1989). Recently, it has been proposed that angiostatic steroids may exert their inhibitory effects on angiogenesis by increasing the synthesis of plasminogen activator inhibitor type-1 by endothelial cells. This, in turn, inhibits plasminogen activator (PA) activity and therefore plasmin generation, which is essential for the invasive aspect of angiogenesis (Blei *et al.*, 1993).

It is interesting to note that dexamethasone produced a drastic suppression of angiogenesis in the sponge model, while medroxyprogesterone was totally without an effect. In contrast, locally applied medroxyprogesterone has previously been shown to inhibit the angiogenesis induced by rabbit V2 carcinoma in rabbit cornea more potently than dexamethasone without heparin treatment (Gross *et al.*, 1981). In the study of Blei *et al.* (1993), the degree of PA inhibition by a variety of angiostatic steroids did not uniformly correlate with their efficacy obtained in the CAM assay (Crum *et al.*, 1985). For example, dexamethasone and medroxyprogesterone were almost equipotent at inhibiting PA activity in cultured endothelial cells. However, 17-α-hydroxyprogesterone, a potent angiostatic on the CAM, was much weaker than dexamethasone and medroxyprogesterone in the PA assay. Such discrepancies are probably related to the differences in assay systems.

In addition to the profound inhibition of sponge-induced angiogenesis, local administration of dexamethasone also caused a dose-dependent atrophy of immune organs such as spleen and thymus, indicative of immunosuppression. In contrast, the angiostatic steroids U-24067 and tetrahydro-S did not produce such systemic effects, suggesting that they function as direct angiogenic inhibitors and not as immunosuppressive and/or anti-inflammatory agents. It might be argued that the anti-angiogenic action of dexamethasone is dependent on its immunosuppressive activity. However, we have previously observed that dehydroepiandrosterone (DHEA, Blauer *et al.*, 1991) prevented the dexamethasone-induced atrophy of spleen and thymus (Hu & Fan, 1992), without modifying the anti-angiogenic action of dexamethasone. Histological studies of sponge implants showed that DHEA produced no apparent effect on leucocyte infiltration. In our recent experiments, spleen cells isolated from animals receiving only dexamethasone responded very poorly to mitogens such as concanavalin A. In contrast, spleen cells from animals receiving DHEA alone, or the combination of dexamethasone and DHEA, showed an active proliferative response when challenged with concanavalin A (Hu & Fan, unpublished studies). Collectively, these data indicate that, in this model at least, dexamethasone can retain its anti-angiogenic activity even when its immunosuppressive actions are blocked by DHEA. It is also noteworthy that daily treatment of rats with cyclosporin A (3, 10 or 30 mg, i.p.) produced a dose-dependent atrophy of spleen and thymus, yet was unable to inhibit sponge-induced angiogenesis (Hu & Fan, unpublished data). Thus, immunosuppressive agents need not necessarily possess anti-angiogenic activity. Likewise, non-steroidal anti-inflammatory drugs such as indomethacin and BW755C (2.5 and 25 μg per sponge per day, respectively) were able to block eicosanoid generation in vascularised sponges, but had no effect on angiogenesis (Smither & Fan, 1991).

In conclusion, the anti-angiogenic activity of angiostatic steroids and anti-inflammatory steroids in the rat sponge model is independent of their ability to reduce the production of TNF-α and IL-6. Unlike dexamethasone (and hydrocortisone), the angiostatic steroids U-24067 and tetrahydro-S did not produce systemic effects on immune organs such as spleen and thymus. Consistent with the earlier results of their inhibitory actions on endothelial cells *in vitro* (Sakamoto *et al.*, 1987; Stokes *et al.*, 1990; Blei *et al.*, 1993), our data strongly support the view that angiostatic steroids function as direct angiogenic inhibitors *in vivo*. At the doses studied, however, it is not possible to separate clearly the anti-inflammatory activity from the anti-angiogenic activity of the glucocorticoids. Although dexamethasone is often used in the treatment of chronic inflammatory diseases, with prolonged administration, it also causes many unwanted side effects such as osteoporosis and suppression of the response to infection or injury. We envisage that future development of drugs based on U-24067 may produce a new generation of therapeutics for the management of angiogenic diseases without concomitant immunosuppression.

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