The Optimal Type, Dosage and Timing of Glucocorticoid Administration for the Treatment of Hemangioma In Vitro

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ABSTRACT

Using our in vitro model, we previously demonstrated the variable effects of different glucocorticoids on cultures of hemangioma biopsies from two patients. The present study was designed to investigate the effects of five commonly used glucocorticoids administered to cultures from the hemangioma biopsies of three patients at different concentrations and at different time points of culture.

Interindividual variation in the effects of glucocorticoids on capillary growth was observed.

Triamcinolone 12 μM inhibited capillary growth in cultures from two out of three patients. Triamcinolone 4 μM stimulated cultures from all patients tested. Dexamethasone 12 μM inhibited cultures from all patients. Up to day 9, dexamethasone 4 μM inhibited cultures from all patients tested. Methylprednisolone 12 μM and 4 μM had little inhibitory effect on cultures derived from all three patients. Betamethasone 12 μM inhibited cultures from two patients but stimulated cultures from another. Betamethasone 4 μM inhibited cultures from all patients tested. Hydrocortisone 12 μM inhibited cultures from one patient and had a variable effect on cultures from the other two. Hydrocortisone 4 μM inhibited cultures from all patients tested until day 9, when cultures from one patient were stimulated.

These in vitro results indicate that dexamethasone may be the steroid of choice for treating hemangioma as it exhibits the least interindividual variation.

Some of the factors considered important for hemangiogenesis were examined. Transcription of fibroblast growth factor-2 was variably affected by different steroids. Transcripts of interleukin-6 were decreased by all glucocorticoids at all concentrations. Transcription of transforming growth factor-β_1 was variably affected. Transcription of clusterin/apo-

LipoproteinJ was increased by all steroids at all concentrations except triamcinolone in cultures from one patient. Transcription of mitochondrial cytochrome *b* was increased by triamcinolone, betamethasone, and methylprednisolone at 12 μM in cultures from one patient, but was reduced by all steroids in those from another.

Glucocorticoids probably modulate hemangiogenesis by altering the transcription of some of the modulators studied. The variable effects reported here may account for the interindividual variation in response to steroids observed clinically.

INTRODUCTION

Hemangioma is a primary tumor of the microvasculature in which there is initial rapid endothelial cell proliferation followed by slow spontaneous regression.[1-4] Most hemangiomas are harmless cutaneous lesions, however, approximately 10% require intervention during the proliferative phase because they threaten life, function, or cause tissue distortion and/or destruction.[5-7] Although various therapeutic modalities have been described, the mainstay treatment in the proliferative phase is pharmacological therapy, with high-dose systemic or intralesional glucocorticoids being the first choice. A dramatic response is observed in 30% of cases, equivocal results are seen in 40%, while continued growth occurs in the remainder.[5-7] This variable interindividual response to steroids is unexplained. Furthermore, the optimal type, dosage, and frequency of steroid administration are largely empirical and do not have a sound scientific basis.[8]

The mechanism of action of steroids in the regression of hemangioma is largely unknown. Cytokines and growth factors are important regulators in the development of hemangioma.[3,4,9-12] Apoptosis has been shown to increase five-fold in hemangioma undergoing regression[13] although the specific factors involved have not been identified. Mitochondrial genes are associated with cell senescence and have been proposed to play an important role in apoptosis.[14] We have previously reported alteration of transcription of mitochondrial cytochrome (mt cyt) *b* in spontaneous and triamcinolone-induced regression of hemangioma.[14-16] We also have demonstrated up-regulation of clusterin/apoLipoproteinJ (clust/apoJ) in spontaneous regression of hemangioma.[17] This protein has been shown to induce apoptotic activity.[3] Employing our in vitro model,[3-18] we have recently reported some of the variable effects of triamcinolone and four other commonly-used glucocorticoids on angiogenic cytokines, mt cyt *b* and clust/apoJ.[8] This present investigation evaluates the in vitro effects of five commonly used glucocorticoids at different concentrations on tissue cultures of hemangioma biopsies over time.

MATERIALS AND METHODS

Experimental Protocol

Biopsies of hemangioma were obtained from three patients (Table 1) in accordance with the protocol approved by the Wellington Ethics Committee. Culture of the biopsy samples was initiated and maintained using the in vitro model described by us.[3,18] In essence, the tissues were rinsed with sterile phosphate-buffered saline immediately after excision and 1 mm^3 pieces of tissue were embedded in fibrin gel in a 24-well culture plate and allowed to grow at 37˚C in serum-free MCDB131 medium and a 3% CO_2/97% air humidified environment. The medium of the culture wells was then supplemented with one of five glucocorticoids: triamcinolone, dexamethasone, methylprednisolone, betamethasone, or hydrocortisone. These were added at the start of culture to a final concentration of 12 μM (Table 1). Steroids were not added to the control wells. Because an earlier report indicates

Table 1. Steroid Administration to Cultures Derived from Three Different Hemangioma Biopsies

Patient	Age (months)	Sex	Glucocorticoids	Concentrations (μM)		Days in Culture
			Triamcinolone	12	4	12
			Dexamethasone	12	4	12
A	8	F	Methylprednisolone	12	4	12
			Betamethasone	12	4	12
			Hydrocortisone	12	4	12
			Triamcinolone	12	4	12
			Dexamethasone	12	4	12
B	13	F	Methylprednisolone	12	4	12
			Betamethasone	12	4	12
			Hydrocortisone	12	4	12
			Triamcinolone	12	2.5	12
			Dexamethasone	12	0.5	12
C	20	F	Methylprednisolone	12	3	12
			Betamethasone	12	0.5	12
			Hydrocortisone	12	12	12

Table 2A. Effects of Different Steroids at 12 μM and 4 μM on Gene Expression in Cultures Derived from Patient B at Day 12

Genes	Controls	Triamcinolone		Dexamethasone		Methylprednisolone		Betamethasone		Hydrocortisone
		12 μM	4 μM	12 μM	4 μM	12 μM	4 μM	12 μM	4 μM	12 μM
VEGF	0	0	0	0	0	0	0	0	0	0
FGF	2	0.5	2	3	1	2	1	1	2	2
IL-6	3	1.5	2	0	0	2	0.5	0.5	1	0
TGF-β_1	2	3	3.5	3	1.5	3.5	2.5	2	2	3
Clust/apoJ	2	2	3	3	2	3	2	2.5	2	3
Mt cyt *b*	2	3	3	1.5	0	3	1.5	3	2.5	2

*VEGF indicates vascular endothelial growth factor; FGF-2, fibroblast growth factor-2; IL-6, interleukin-6; TGF-β_1, transforming growth factor β_1; clust/apoJ, clusterin/apoLipoproteinJ; met cyt *b*, mitochondrial cytochromeb. Transcript expression was semi-quantitated adter gel documentation (0=no expression, 4=maximal expression).

that steroids also exhibit antiangiogenic activity at low concentrations,[19] a further set of experiments was done in which all five glucocorticoids were added to a final concentration of 4 μM to cultures from two patients (A and B) (Table 1). For cultures from patient C, all five glucocorticoids were added at concentrations calculated to be equally potent to 12 μM hydrocortisone[20] and subsequently analysed for gene transcription as described below. A minimum of four wells was used for each treatment type, including controls. The cultures were maintained for up to 12 days. Any visibly contaminated well was immediately destroyed as described.[3]

Assessment of Capillary Growth

The capillary growth in each well was recorded at least three times during the culturing period by capturing images with a Pixera digital camera described previously.[3,18,21] The neovascular area in each digitized image was outlined and calculated using the NIH Image Program (NIH, Bethesda, MD, USA).[3,18]

The capillary growth of each culture was expressed as the ratio of the area occupied by the neovessels to the area of hemangioma tissue from which they emanated.[3,8,18]

Table 2B. Effects of Different Steroids at 12 μM on Gene Expression in Cultures Derived from Patient C at Days 6 and 12*

Time Points	Genes	Controls	Triamcinolone	Dexamethasone	Methylprednisolone	Betamethasone	Hydrocortisone
	VEGF	0	0	0	0	0	0
	IL-6	2	3	3.5	4	3.5	4.5
	TGF-β_1	2	2	3.5	2	3	3
Day 6	Clust/apoJ	1	2	3	3	3	2
	Mt cyt *b*	1	1	2.5	2.5	2.5	3
	VEGF	0	0	0	0	0	0
	IL-6	4	1	1	1	2	2
	TGF-β_1	3.5	1	2	1	2.5	2
Day 12	Clust/apoJ	1	2.5	2	2	1.5	1.5
	Mt cyt *b*	3	0.5	0.5	-	1	0.5

*VEGF indicates vascular endothelial growth factor; FGF-2, fibroblast growth factor-2; IL-6, interleukin-6; TGF-β_1, transforming growth factor β_1; clust/apoJ, clusterin/apoLipoproteinJ; met cyt *b*, mitochondrial cytochromeb. Transcript expression was semi-quantitated adter gel documentation (0=no expression, 4=maximal expression).

Table 2C. Effects of Different Steroids at Concentrations of Equal Potency to 12 μM Hydrocortisone on Gene Expression in Cultures Derived from Patient C at Day 12*

Genes	Controls	Triamcinolone 0.5 μM	Dexamethasone 3 μM	Methylprednisolone 0.5 μM	Betamethasone 2.5 μM	Hydrocortisone 12 μM
VEGF	0	0	0	0	0	0
IL-6	4	3.5	1.5	3	2	2
TGF-β_1	3.5	4	2.5	3.5	2	2
Clust/adoJ	1	1	1	1.5	1.5	1.5
Mt cyt *b*	3	3	1.5	2	0.5	0.5

*VEGF indicates vascular endothelial growth factor; FGF-2, fibroblast growth factor-2; IL-6, interleukin-6; TGF-β_1, transforming growth factor β_1; clust/apoJ, clusterin/apoLipoproteinJ; met cyt *b*, mitochondrial cytochromeb. Transcript expression was semi-quantitated adter gel documentation (0=no expression, 4=maximal expression).

Analysis of Gene Transcription

At the end of the experiments for patients B and C, at least three culture wells from each treatment type were harvested for RNA isolation using Trizol (Gibco, Life-Technol., Auckland).[15-17] Gene transcription analysis with reverse transcriptase-polymerase chain reaction was undertaken, as detailed previously, using primers for vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), interleukin-6 (IL-6), transforming growth factor-β_1 (TGF-β_1), mt cyt *b*, and clust/apoJ.[15-17] Transcript expression was semiquantitated after gel documentation (0 = no expression to 4 = maximal expression) and tabulated as described.[8]

Gene expression for cultures from patient B was assessed at two concentrations of glucocorticoids on day 12 of culture. Gene expression for cultures from patient C was assessed at a variety of concentrations (Table 2C) and at two time points: day 6 prior to any noticeable variation in capillary growth and day 12 when the culture was terminated.

Statistical Analysis

The mean of the ratios of the area occupied by the neovessels to the area of hemangioma tissue was calculated for cultures from each patient, for each treatment type and for each time point at which capillary growth was measured. These mean values are plotted as a function of time in culture in Figure 1. The sample size depended on how many culture wells were contaminated and subsequently excluded from data analysis.

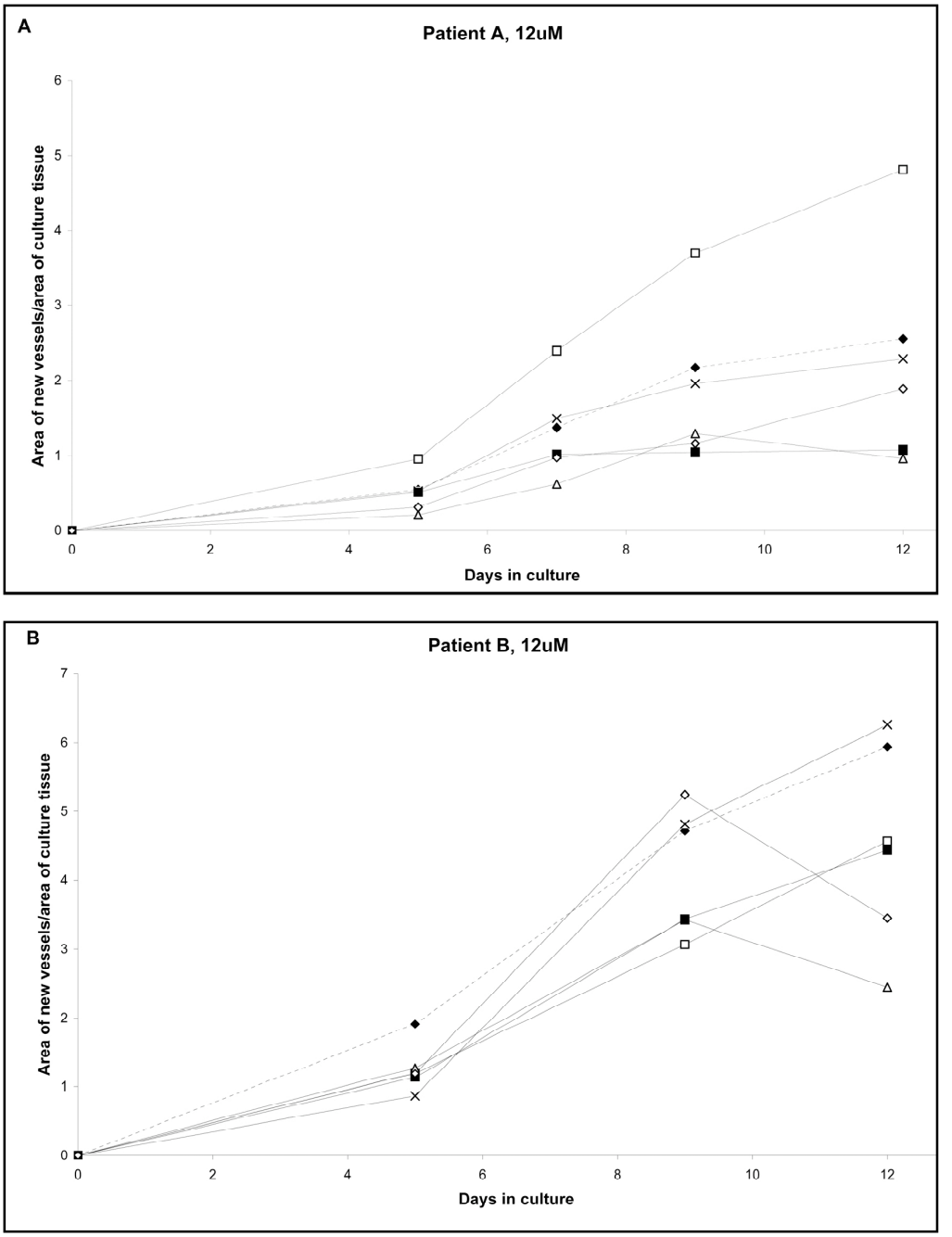

Figure 1 A & B. In vitro effects of five glucocorticoids on capillary growth of cultures derived from hemangioma biopsies from patients A, B, and C at concentrations shown. ◆, control; □, triamcinolone; ▯, dexamethasone; ▯, methylprednisolone; ■, betamethasone; ◇, hydrocortisone.

RESULTS

Capillary Growth

All cultures of all three hemangioma biopsies grew well in the serum-free environment. Distinct capillary growth was observed from day 5. On day 5, the glucocorticoids had no noticeable effect on capillary growth. From day 7, inhibition of capillary growth by some of the glucocorticoids was observed. Hence, compari-

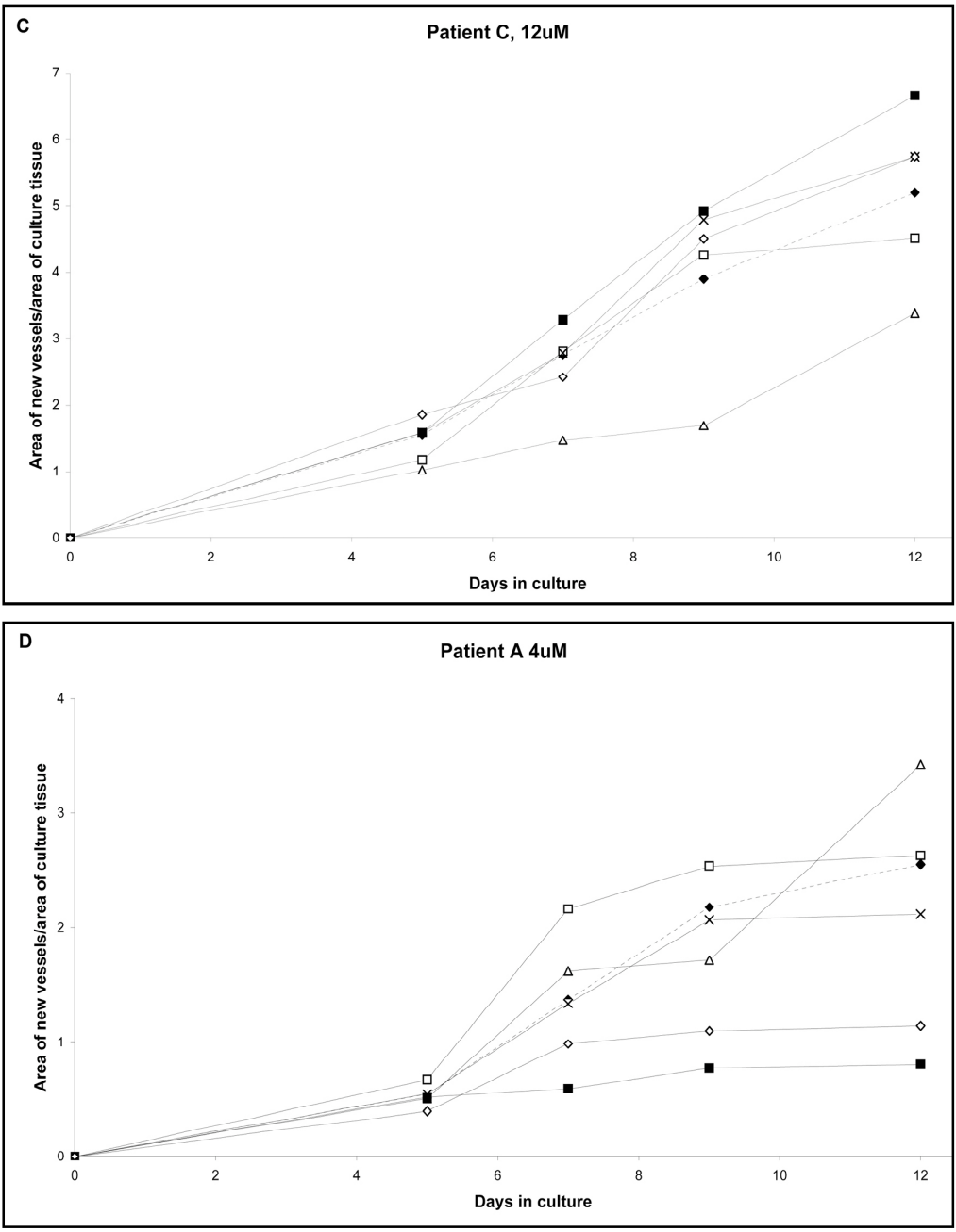

Figure 1 C & D. In vitro effects of five glucocorticoids on capillary growth of cultures derived from hemangioma biopsies from patients A, B, and C at concentrations shown. ♦, control; □, triamcinolone; ▯, dexamethasone; ▯, methylprednisolone; ■, betamethasone; ◇, hydrocortisone.

son of the ratios of area of new vessels to area of culture tissue was made at intervals between day 5 and termination of the experiment on day 12 (Figure 1).

Steroid Treatment at 12 µM (Patients A, B and C—Figures 1 A-C). Results of treatment with different glucocorticoids at 12 µM show a degree of interindividual variation. On day 12, triamcinolone inhibited capillary growth in cultures from patients B and C, but stimulated growth in cultures from patient A.

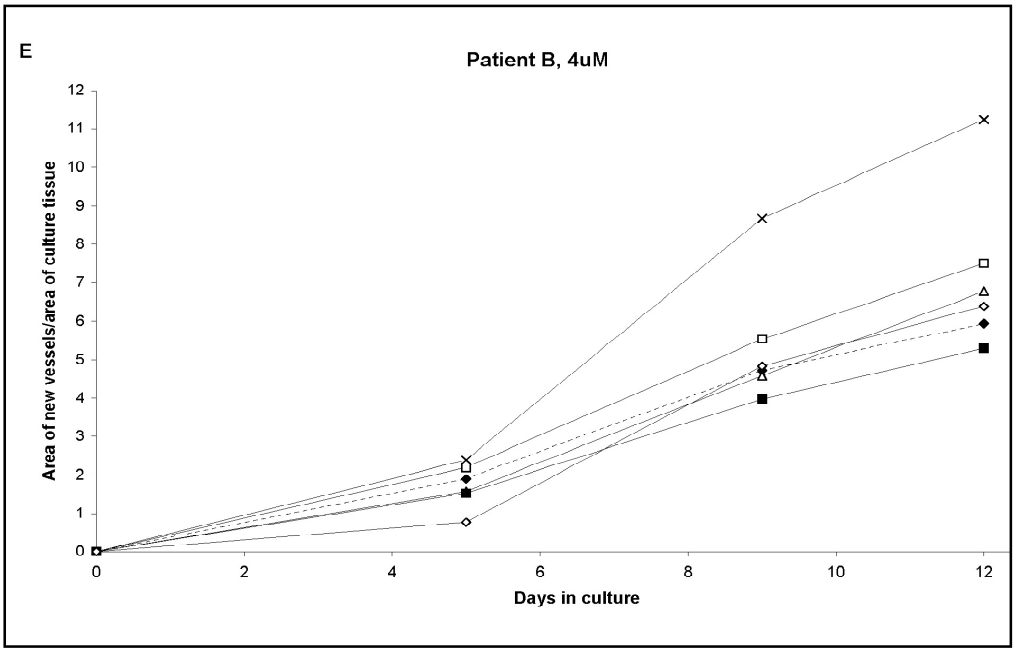

Figure 1 E. In vitro effects of five glucocorticoids on capillary growth of cultures derived from hemangioma biopsies from patients A, B, and C at concentrations shown. ♦, control; □, triamcinolone; ▯, dexamethasone; ▯, methylprednisolone; ■, betamethasone; ◇, hydrocortisone.

Dexamethasone inhibited capillary growth in all cultures. Methylprednisolone had negligible effect on capillary growth in cultures from all three patients over time. Betamethasone inhibited growth in cultures from patients A and B but stimulated growth in cultures from patient C. Hydrocortisone inhibited growth in cultures from patient A but had a variable effect on cultures from patients B and C at different time points.

Comparison of Steroid Treatment at 12 µM and at 4 µM (Patients A and B—Figures 1A-E). Whereas triamcinolone 12 µM showed interindividual variation in its effects, triamcinolone 4 µM stimulated capillary growth from all cultures. Dexamethasone 12 µM inhibited all cultures for the duration of the experiments but at 4 µM that inhibition was lost after day 9. Methylprednisolone 12 µM had very little effect on cultures from patients A and B. With methylprednisolone 4 µM cultures from patient A were not notably affected but cultures from patient B were stimulated. Betamethasone 12 µM inhibited cultures from patient B while stimulating cultures from patient A. However, betamethasone 4 µM inhibited cultures from both patients. Cultures treated with hydrocortisone 12 µM and 4 µM are inhibited except on day 9, when cultures from patient B, treated with this steroid at 4 µM, were stimulated.

Gene Transcription

Gene Expression at Two Different Concentrations of Steroid on Day 12 (Patient B—Table 2A). VEGF transcripts were not detected in either the controls or steroid-treated cultures at any concentration. FGF-2 transcript levels were reduced by triamcinolone 12 µM and betamethasone 12 µM on day 12. This was associated with inhibition of capillary growth in cultures from patient B. Conversely dexamethasone 12 µM increased transcription of FGF-2, while methylprednisolone 12 µM and hydro-

cortisone 12 μM had no effect. IL-6 transcription was markedly down-regulated by all five glucocorticoids compared to controls. However, decreased levels of IL-6 transcription were not consistently associated with capillary growth inhibition (Figure 1A). Transcription of TGF-β_1 was up-regulated by all steroids at 12 μM except for betamethasone. At 4 μM, TGF-β_1 was only up-regulated by triamcinolone and methylprednisolone. Dexamethasone 4 μM inhibited TGF-β_1 transcription. Betamethasone did not alter transcription of TGF-β_1 at either concentration.

All steroids at 12 μM, except triamcinolone, increased transcription of clust/apoJ. Transcription of mt cyt *b* was increased by triamcinolone and betamethasone 12 μM and 4 μM at both concentrations but was increased by methylprednisolone 12 μM only. Transcription of mt cyt *b* was unaffected by hydrocortisone 12 μM and was reduced by dexamethasone at both concentrations.

Gene Expression at Different Time Points with Steroid Treatment at 12 μM (Patient C—Table 2B). Gene transcription was assessed on days 6 and 12 in cultures from patient C. VEGF transcripts were not detected at either time points. On day 6, all glucocorticoids increased the transcription of IL-6 compared to control, but transcripts levels were reduced on day 12. On day 6, transcription of TGF-β_1 was increased by dexamethasone, betamethasone, and hydrocortisone, but was unaltered by triamcinolone and methylprednisolone. On day 12, transcription of TGF-β_1 was decreased by all steroids. On days 6 and 12, all steroids markedly increased the transcription of clust/apoJ compared to controls. On day 6, all steroids except triamcinolone, increased mt cyt *b* transcription, which was subsequently reduced by all steroids on day 12.

Gene Expression with Steroids at Concentrations of Equal Potency to Hydrocortisone 12 μM (Patient C—Table 2C). Cultures from patient C were treated with steroids at concentrations of equal potency to hydrocortisone at 12 μM. VEGF mRNA was not detected in any of the experiments. Expression of IL-6 was decreased in cultures treated with the different steroids at concentrations of equal potency to hydrocortisone at 12 μM. TGF-β_1 transcription was reduced by all glucocorticoids except triamcinolone by which it was increased.

Transcripts of clust/apoJ were increased with all steroids except triamcinolone, which had no effect. Transcript expression of mt cyt *b* was lowered by all steroids except triamcinolone with which it was unchanged.

DISCUSSION

Approximately 10% of hemangiomas require intervention during the proliferative phase with different agents such as steroids, interferon-alpha, and recombinant platelet-derived growth factor.[7,22,23] High-dose systemic or intralesional glucocorticoids are the first-line pharmacological treatment.[5-7] However, the optimal type, dosage, and frequency of steroid administration are unknown. We have used our own in vitro model[3,18] to study the effects of different steroids on hemangioma.

Angiostatic activity has been observed among many steroid classes. A good correlation between the angiostatic efficacy of 15 different steroids tested in the chick chorioallantoic membrane and the rabbit lipopolysaccharide-induced corneal pocket models of neovascularization has been reported.[24] However, their mechanism of action is largely unknown.

In a recent study using bovine choroidal endothelial cell culture, triamcinolone at concentrations of 100, 150, and 300 mg/L has been shown to inhibit

capillary tube formation after five days.[25] Topical administration of triamcinolone exhibits a stronger inhibitory effect on neovascularization than systemic administration in a rabbit ear model.[26] Our results also show that triamcinolone 12 μM inhibits capillary growth in cultures from two of the three patients and this effect is apparent from day 7 onwards.

It is known that hydrocortisone can be converted to a potent angiogenic inhibitor by co-administering it with heparin or with a sulfated-cyclodextrin.[27] In the absence of exogenous heparin, hydrocortisone does not inhibit angiogenesis.[28] However, a subsequent study over a 20-day period shows that daily local injections of hydrocortisone in a basal sponge-induced angiogenic model produce a dose-dependent (0.5, 5, and 50 mg/sponge) inhibition of neovascular development.[29] Systemic administration with hydrocortisone (2, 10, and 50 mg/kg) has been shown to be less effective at inhibiting angiogenesis, and this effect is not sustained until day 20.[29] We also have observed a similar inhibition of capillary growth by hydrocortisone in the early stages of culture, although this effect is lost at later time points. This suggests that repeated doses of this steroid may be required for suppressing capillary growth and in the treatment of hemangioma.[8]

Dexamethasone and methylprednisolone at very low and nontoxic subcutaneous doses inhibit angiogenesis.[30] Daily local injection of dexamethasone 0.5 μg/sponge inhibits basal sponge-induced angiogenesis almost completely. At higher doses (5 and 50 μg/sponge), dexamethasone does not produce additional inhibition of angiogenesis. Our results demonstrate that dexamethasone 12 μM and 4 μM inhibited capillary growth in cultures from all three patients. Methylprednisolone 12 μM and 4 μM had no inhibitory effect on cultures from all three patients. Betamethasone 12 μM inhibited capillary growth in cultures from two patients (A and B) but not the third patient (C), although at 4 μM it was inhibitory in cultures from all three patients. Hydrocortisone 12 μM inhibited cultures from one patient (A) and had a variable effect on cultures from the other two (B and C). At 4 μM, it inhibited cultures from all patients tested until day 9, when cultures from one patient (B) were stimulated. Hence the effects of all five glucocorticoids exhibit a glucocorticoid-specific interindividual variation in capillary inhibition. This suggests that the same glucocorticoid may elicit different responses from different patients with hemangioma and the choice of the type of steroid is therefore important. Our in vitro results indicate that dexamethasone may be the steroid of choice for treating hemangioma as it exhibits the least interindividual variation.

Although a number of studies indicate their effect on cytokines and growth factors,[31] the mechanism of action of glucocorticoids in inhibiting neovascularization remains unclear. An earlier report shows that the capacity of corticosteroids to prevent new blood vessel formation is mediated primarily through abolishing the expression of VEGF.[32] Since VEGF transcripts are not detected in either the controls or steroid-treated cultures from as early as day 6, we hypothesize that either VEGF transcription may have occurred transiently during the initiation of capillary growth or that this factor is not essential for hemangiogenesis.[8] Our observations suggest that glucocorticoids alter the transcription of factors other than VEGF to promote regression of hemangioma.[16] Transcription of FGF-2, another important angiogenic cytokine, is altered by glucocorticoids, but its down-regulation is not associated with inhibition of capillary growth.[8]

An earlier report indicates that daily doses of dexamethasone at 5 µg/sponge, inhibits angiogenesis and produces a marked reduction in the levels of TNF-α and IL-6 on day 14.[29] In contrast, hydrocortisone does not influence the levels of TNF-α and IL-6. Our results show that all five glucocorticoids tested cause an increase in IL-6 transcription on day 6, followed by a marked reduction in transcript levels by day 12.

TGF-β1 transcription is unchanged by triamcinolone and methylprednisolone and is elevated by dexamethasone, betamethasone, and hydrocortisone on day 6, but is markedly reduced on day 12 by all five glucocorticoids at 12 µM. Both IL-6 and TGF-β1 transcription are not specifically associated with capillary inhibition. Hence we concur with others[16] that the antiangiogenic activity of steroids is independent of their ability to alter the production of cytokines such as IL-6, TNF-α and TGF-β1.

The expression of clust/apoJ, an apoptotic factor, is important in the regression of hemangioma.[17] It is increased by all five glucocorticoids at all concentrations on day 6 and 12 except at lower concentrations of triamcinolone.

Mitochondrial genes are associated with cell senescence and have been regarded to play an important role in apoptosis.[14] We previously reported the alteration of the transcription of mt cyt *b* both in spontaneous and triamcinolone-induced regression of hemangioma.[15,16] In cultures from patient C, mt cyt *b* expression is increased by all steroids except triamcinolone 12 µM on day 6. However, its expression is decreased by all steroids except triamcinolone 2.5 µM on day 12. In cultures from patient B, increased transcription of mt cyt *b* by triamcinolone 12 µM and betamethasone 12 µM is associated with inhibition of capillary growth. This suggests that these two glucorticoids may mediate their actions by affecting the normal regulation of apoptosis leading to inhibition of capillary growth or regression of hemangioma.

Changes in the transcription of the various cytokines at different time points following steroid treatment, as well as the variation in transcription levels with different concentrations of steroids, suggest that glucocorticoids regulate cytokines differentially. Our results also indicate that transcription of clust/apoJ and mt cyt *b* is affected by the dosage and timing of steroid administration.

This study shows a differential response of individual hemangiomas to glucocorticoids, which may exert their effects on different modulators. Our results underscore the importance of the choice of the type, dosage, and frequency of steroid administration in treating hemangioma and other neovascular diseases.

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