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Short communication

Cortisone inhibition of tumor angiogenesis measured by a quantitative colorimetric assay in mice

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Summary. A simple and quantitative angiogenesis assay was developed. Using this assay, the angiostatic effect of cortisone acetate (CA) on three murine tumors was studied. Tumor cells were inoculated i.d. into the syngeneic or heterogeneic hosts (day 0) and the degree of angiogenesis was quantitated on day 3 by measuring the tumor vascular volumes using an Evan's blue perfusion technique. CA treatment (250 mg/kg for 3 days) significantly suppressed tumor angiogenesis; however, the degree of angiostatic effect was influenced by the tumor types and by the mouse strain used. MBT-2 bladder cancer angiogenesis was suppressed by 77%–80% of controls in C3H/HeN and C57Bl/6 mice, whereas MBT-2 angiogenesis in BALB/c mice was significantly less suppressed by CA (65% inhibition) as compared with values obtained for C3H mice. B16 melanoma or Line-1 lung-cell carcinoma-induced angiogenesis was suppressed by 57%–66% in their syngeneic or heterogeneic hosts. The combined administration of CA and heparin (Sigma; 1,000 units/ml in drinking water) did not influence the outcomes. The data suggest that host factor(s) and tumor factor(s) influenced the expression of CA angiostatic activity. This colorimetric assay enabled a quantitative estimation of the degree of angiogenesis in mammalian animals.

Introduction

In 1971 Folkman [2] introduced the concept of "antiangiogenesis" for cancer treatment. Since then a number of substances have been reported to inhibit angiogenesis (see [11]). However, the effect of antiangiogenesis may not necessarily be synonymous with the antitumor effect, as evidenced by the fact that very few angiostatic substances demonstrate an ability to suppress established solid tumors in animals. The synergistic effect of cortisone and heparin was first described by Folkman et al. [4]. Local administration of cortisone with heparin halts tumor angiogenesis in rabbit cornea, and systemic administration of both agents inhibits the growth of palpable tumors in mice. Cortisone alone and heparin alone show little or no effect [4]. These observations were confirmed by one laboratory [12], whereas others have reported that cortisone alone (without heparin) suppresses tumor growth [7, 8, 10, 13] and inhibits tumor angiogenesis in mice [7]. We speculated that the apparent discrepancy between the angiostatic and the antitumor effects of cortisone in mice was attributable to the different animal strains used [7], although this could not be verified due to the lack of an appropriate method. Therefore, a simple and quantitative angiogenesis assay was developed; using this assay, the antiangiogenic effect of cortisone on three different murine tumors was studied.

Materials and methods

Mice, tumors and treatment protocol. Inbred female C3H/HeN (C3H), BALB/c, and C57Bl/6 (C57) mice were purchased from Charles River Laboratory (Pitsburgh, Pa.). Mice weighing 20–22 g were used for the experiments. The tumor cells used included MBT-2 bladder cancer (H-2^k) [7], Line-1 (L1) lung-cell carcinoma (H-2^d) [9], and B16-F10 melanoma (H-2^b; obtained from Dr. K. Tsukamoto, NCI, Bethesda, Md.). The cells were maintained by in vitro culture and cells from the 3rd–7th in vitro passages were used for the experiments. Routine assay of cell lines for *Mycoplasma* using agar culture [7] was negative. Tumor cell suspensions were prepared by short-term trypsinization of subconfluent flasks, which were washed once with Dulbecco's modified Eagle's medium (DO; Sigma, St. Louis, Mo.); viable cells were then counted (trypan blue exclusion method) and suspended in DO.

Cortisone acetate (CA) (Sigma) was suspended in 0.9% NaCl solution containing 0.5% ethanol at 25 mg/ml. CA was injected s.c. at 250 mg/kg for 3 days. Heparin (Sigma; H-7005) was given in drinking water at 1,000 units/ml for 3 days. Treatment was initiated 4 h after tumor inoculation, when mice were awakened from anesthesia. Control mice were given no treatment.

Colorimetric angiogenesis assay. With mice under general anesthesia consisting of sodium pentobarbital (1.4 mg/mouse, i.p.), tumor cells

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Table 1. Tumor angiogenesis in syngeneic and heterogeneic mice

Tumor	Degree of angiogenesis (tumor vascular volume; μl/6.4 mm²)		
	C3H/HeN hosts	C57Bl/6 hosts	BALB/c hosts
MBT-2	1.58±0.23	1.57±0.59	1.44±0.49
B16	1.44±0.28	1.10±0.33	0.87±0.23[a]
L1	1.83±0.63	1.78±0.28[b]	2.19±0.31[b, c]

The degree of angiogenesis was measured on day 3. The tumor vascular volume represents the blood volume of two tumored skin disks (total of 4×10^6 tumor cells) extracted by the mean background blood volume. The background (6.4 mm² normal skin) blood volume was 0.28±0.083 μl in C3H mice, 0.35±0.071 μl in C57Bl mice, and 0.27±0.095 μl in BALB/c mice. Each data point represents the mean ± SD of 8–12 samples

[a] $P < 0.05$ vs B16 in C3H mice

[b] $P < 0.05$ vs B16 in C57 and BALB/c mice

[c] $P < 0.05$ vs MBT-2 in BALB/c mice

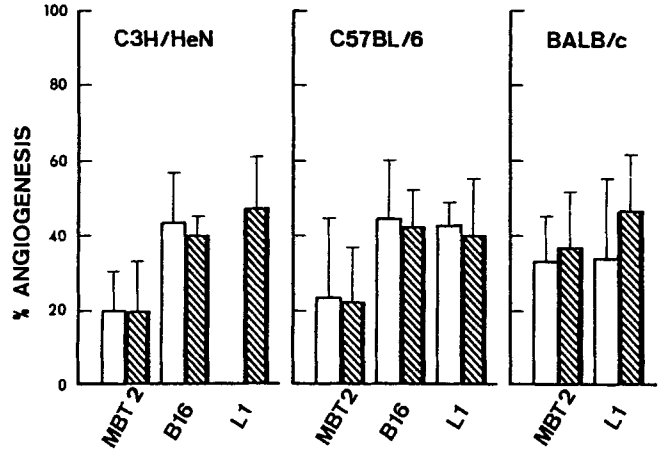

Fig. 1. Influence of cortisone acetate (CA) with or without heparin treatment on tumor angiogenesis. Mice were given CA alone (250 mg/kg s.c.) (*open column*) or CA plus heparin (1,000 units/ml in drinking water) (*shaded column*) for 3 days beginning on day 0. Tumor vascular volume (angiogenesis) in untreated mice is shown in Table 1. Each column represents the mean + SD of 8–12 samples. $P < 0.05$ for MBT-2 vs B16 and L1 in C3H mice and for MBT-2 in C3H mice vs MBT-2 in BALB/c mice

were inoculated i. d. on day 0, at eight sites of each recipient (bilaterally in the upper and lower abdominal skin and similarly on the back. Each inoculum comprised 2×10^6 cells (unless otherwise specified) in 50 μl, injected with a 26-gauge needle. The degree of angiogenesis was assessed by measuring the tumor vascular volume on day 3. Through the tail vein, mice were injected i. v. with 0.25 ml 1% Evan's blue (Sigma) in 0.9% NaCl solution [1]; 2 min later they were killed by cervical dislocation. A mid-vertical incision was then made, and the skin flap was raised bilaterally. Filter papers were placed on the inner surface of the skin, and the skin flaps were dissected. The inoculations were then punched out using a paper punch (with a 6.5 mm diameter, or 33.2 mm²). The use of filter paper facilitated the procedures. Two hemilateral sites of skin disks were pooled, placed in a 1.5-ml polypropylene centrifuge tube, and cut into small pieces; i. e., the eight implant sites on a single mouse were paired to represent four samples. Similarly, normal skin (background) was obtained from non-tumored mice. The tissue was suspended in 1.5 ml sodium sulfate/acetone solution (0.5% Na_2SO_4 $1OH_2O$/acetone = 2/3, v/v) and kept at room temperature for 24 h with occasional shaking [5]. Absorbance of the supernatant was read at 620 nm.

The blood standard, obtained from a subset of four mice, was used throughout the experiments. At 2 min after dye injection, the mice were bled from the femoral artery while under ether anesthesia and the blood was pooled (600 μl/mouse) and suspended in substrate at 2 μl blood/ml; 24 h later, the supernatant was separated in aliquots and stored at −135° C until use. The detection limit was approximately 0.03 μl blood/ml. In our preliminary experiments, blood was obtained from individual mice (through the axillary artery or by cardiac puncture) and the inter- and intraassay variations of the blood standard were found to be extremely small. Based on this observation, the pooled blood from the subset of mice was used for our standard to minimize labor.

The results were expressed as either tumor vascular volume (blood volume of tumored skin extracted by mean background blood volume) or percentage of angiogenesis. The percentage of angiogenesis was determined by the formula

$$(A - B) / (C - B) \times 100,$$

where A, B, and C represent the A_{620} (or blood volume) of the treated tumor, background skin, and control tumor, respectively. Each data point consisted of 8–12 samples, and no data were excluded. Individual data points were obtained from single studies; overall data were obtained from four independent experiments.

Statistic comparison. Data were expressed as the mean ± SD. Statistical comparisons were made by Student's *t*-test, with significant being determined at $P < 0.05$.

Results

The relationship between the number of tumor cells inoculated and the degree of angiogenesis (tumor vascular volume) was determined using MBT-2 tumors in C3H mice. Mice were inoculated with 8×10^5, 1.6×10^6, or 2×10^6 cells per site. The tumor vascular volume (y) was increased in a manner dependent on the log number of cells inoculated (x), as given by the formula
$y = -7.06 + 1.369 \log x$ ($r = 0.997$).

Table 1 summarizes the angiogenic activity of three different tumor cell lines in three different hosts, which were inoculated with 2×10^6 tumor cells per site. The angiogenic activity of L1 carcinoma was significantly higher than that of B16 cells in C57 and BALB/c mice. The MBT-2 cells induced a similar degree of angiogenesis, irrespective of the host. Interestingly, B16 cells were less capable of inducing neovascularization in BALB/c mice than in C3H or C57 mice.

The influence of CA with or without heparin treatment on tumor angiogenesis is shown in Fig. 1. CA treatment significantly inhibited all tumor angiogenesis in the different hosts. However, the degree of angiogenic inhibition was influenced by the tumor type and the mouse strain. MBT-2 angiogenesis was inhibited by 77%–80% of control values in C3H and C57 mice, whereas that in BALB/c mice was significantly less suppressed by CA (65% suppression) as compared with values obtained for C3H mice. CA treatment suppressed B16 and L1 angiogenesis by 57%–66% in syngeneic and heterogeneic hosts. Combined administration of heparin and CA (Fig. 1) did not influence the outcomes.

Discussion

The antitumor efficacy of CA in murine tumor models is varied [4, 7, 10, 12, 13]. In the present investigation, we studied the angiostatic effect of CA on three different tumor models. Reportedly, CA treatment does not suppress the growth of B16 melanoma [4]. In our studies, L1 carcinoma did not respond to CA treatment (unpublished observation), whereas CA treatment significantly suppressed MBT-2 growth [7]. Unexpectedly, the present data demonstrate that CA alone significantly suppressed all tumor angiogenesis in syngeneic hosts; i.e., 57% inhibition of B16 (in C57mice), 66% inhibition of L1 (in BALB/c mice), and 80% inhibition of MBT-2 (in C3H mice) (Fig. 1). Along with the CA antitumor effect, the data suggest that partial or incomplete (57%–66%) inhibition of tumor angiogenesis may have a minimal impact on solid tumor growth. For suppression of solid tumor growth, >66% or nearly 80% inibition of angiogenesis may be required.

Moreover, the data suggest that not only host factor(s) but also tumor factor(s) are responsible for the angiostatic activity of CA. MBT-2 angiogenesis was more profoundly suppressed in C3H mice than in BALB/c mice, suggesting that C3H mice were angiostatically more sensitive to CA than were BALB/c mice. In C3H mice, CA (or CA plus herapin) treatment more profoundly suppressed MBT-2 angiogenesis than B16 or L1 angiogenesis, suggesting that MBT-2 was more sensitive to CA than were L1 or B16 tumors. The lack of a synergistic effect of CA with heparin was probably attributable to either the source of the heparin used [4] or the strain of animal used.

Regarding tumor angiogenesis and the inhibition assay, very few methods are available at present. Individual methods rely on the estimation of the degree of neovascularization around the tumor (or tumor extracts). These assays can be classified into two groups based on the route of administration of test agents. The first group includes chick embryo (chorioallantoic membrane) [2, 3] and rabbit (or rodent) cornea assay [4, 11], which are an analogue of bacterial chemosensitivity tests. Angiogenic substances are implanted into the hosts, test agents are inoculated near the stimulus, and the area of avascular zone or the vascular length is quantitated. This type of assay is useful for screening of angiostatic agents, including the quantitation of the activity. However, from a therapeutic standpoint, these assays may potentially overestimate the activity of certain agents because local drug concentration is higher than drug levels in any other tissue. As capillaries grow toward the stimulus, the drug concentration becomes higher.

The other type is a rodent skin assay. Tumor cells are implanted s.c. or i.d. into rodents, and agiostatic agents are given systemically [6, 12]. This assay enables the assessment of the ability of systemic agents to inhibit angiogenesis at tumor sites. However, comparative study has previously been unsuccessful because of the lack of quantitative and sensitive methods for assessing angiogenic activity. In our experience with dorsal air-sac methods [7] and the so-called i.d. method (unpublished data), we have encountered large inter- and intraassay variations, probably due to the three-dimensional structure of neovascularization. The present data suggest that the additional use of the dye-perfusion technique provides an objective endpoint and diminishes the variations, as evidenced by relatively small SD values (Table 1, Fig. 1). In turn, the sensitivity of the assay was dramatically increased. Moreover, we found that this assay was less labor-intensive and required a relatively small number of animals as compared with conventional murine assays.

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