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Chemotherapy and Antiangiogenesis

Drug-specific, Dose-related Effects

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Dose-response effects of fluorouracil, paclitaxel, doxorubicin, cisplatin, methotrexate, cyclophosphamide and etoposide on $VEGF_{165/164}$-mediated angiogenesis using the rat mesenteric-window angiogenesis assay are reported. VEGF is a pivotal pro-angiogenic factor in most tumors. Microvessel spatial extension, density, pattern formation and segment length were assessed quantitatively and objectively. A single i.v. injection of each drug was given at a low, intermediate or high dose, 7 days before sacrifice. All the drugs elicited significant responses in terms of one or more measured variables. Only paclitaxel, doxorubicin and cyclophosphamide significantly suppressed the overall angiogenic response ($p \leq 0.0001$, $p \leq 0.0002$ and $p \leq 0.05$, respectively), however. Taking toxicity into account, paclitaxel was more potent in inhibition of angiogenesis than the other agents. No clear correlation was found between drug half-life, the degree of toxic effects (in terms of body weight changes) and the antiangiogenic effect. The antiangiogenic effects were distinctly drug specific.

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In recent studies (1, 2) it is reported that the frequent or continuous treatment of mice with s.c.-transplanted tumors with a chemotherapeutic drug (cyclophosphamide or vinblastine) at a low, well-tolerated dose administered at frequent intervals, i.e. 'antiangiogenic' (1) or 'metronomic' treatment (3), exerted much stronger antiangiogenic and anti-tumor effects than conventional, low-frequency, high-dosage treatments. However, the exact role of the anti-angiogenic effect in the observed anti-tumor response cannot be elucidated at present.

In a recent survey of chemotherapeutics as antiangiogenics (4) it was observed that relatively few agents have been shown to alter angiogenesis in vivo. Because the field is still largely in its infancy, the investigators were led to ask whether chemotherapeutics in general are really antiangiogenics in disguise and how we would ever know the answer, as different assays produce different results. Clearly, there is a need to study the antiangiogenic effect of major chemotherapeutic agents *per se*.

The present pilot study was carried out with the aim of characterizing the dose-dependent angiogenesis-modulating features of several standard cytotoxic chemotherapeutic agents using a highly standardized and truly quantitative model. We used a tumor-free, biologically relevant model and studied several objective variables of $VEGF_{165/164}$-mediated angiogenesis, as the influence of chemotherapy on tumor angiogenesis may be tumor and site dependent. $VEGF_{165/164}$, an endothelial cell-specific mitogen and survival factor, is a major angiogenic factor in most tumors (5, 6). VEGF also seems to play a role in angiogenic pathways that are related to other angiogenic factors (7). VEGF was injected i.p. at picomolar, approximately physiologically relevant doses in rats. The cytotoxic drugs were subsequently administered in single i.v. injections at comparatively low, intermediate and comparatively high doses. Seven days later, the response in the mesentery was quantified in terms of microvessel spatial extension, density, pattern formation and microvessel segment length. Discrete drug-specific effects were found.

MATERIAL AND METHODS

Animals

Adult male outbred Sprague-Dawley rats (B & K Universal, Sollentuna, Sweden) were acclimatized to a standardized environment for a minimum of 5 days, fed ad libitum and

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randomly allocated to weight-matched groups with two animals per cage. The rats were kept in a computer-controlled animal room at 20°C with 47% relative humidity and an air turnover rate of 15 times/h. The light was kept on from 7 a.m. to 7 p.m. at an intensity of ~100 lux. At the start of the experiments, the mean body weight varied between 215.7 g and 228.6 g. Body weight was monitored twice a week and the controls increased by 60–68 g a week during the experimental period (Table 2). Weight-gain retardation was regarded as a surrogate evaluation of toxicity, which also included systemic well-being, anorexia and failure to thrive. The animal ethics committee in Göteborg approved the study. The ethical guidelines that were followed met the standards required by the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines (8).

Angiogenesis treatment

Recombinant human $VEGF_{165}$ and recombinant rat $VEGF_{164}$ (293-VE/CF and 564-RV/CF, respectively; R&D Systems, Ltd., Oxon, UK) were diluted to 96 pmole/ml, frozen, thawed and a volume of 5 ml was injected i.p., as described elsewhere (9). $VEGF_{165}$ treatment at this dose, twice daily for 4 1/2 days, i.e. from Monday morning (Day 0) to Friday morning (Day 4), causes a strong angiogenic response (9); the controls received the saline vehicle (infusion solution, Pharmacia). The angiogenic responses to $hrVEGF_{165}$ and $rrVEGF_{164}$ are fully consistent in our system (data not shown). In mouse mesentery microvessels, an i.p. injection of $hrVEGF_{165}$ promotes angiogenesis cascade signaling by inducing the selective expression of Flk-1 and Flt-1 and the tyrosine phosphorylation of specific proteins, such as Flk-1, phospholipase C-gamma and mitogen-activated protein kinase (10).

Since the present test tissue is natively vascularized and lacks significant physiological angiogenic activity, like most normal adult tissues, and is not exposed to surgery, the induction and development of angiogenic responses that resemble those occurring in tissues affected by clinically relevant angiogenic diseases (e.g. tumors) can be studied in this assay.

Chemotherapy

The details of each drug (class, half-life and dose) are presented in Table 1; potential contributions by any active metabolite(s) must also be considered in the comparison between the drugs that were used. Drug dosage was selected on the basis of the published i.v. bolus doses that can be given to rats without obtaining severe toxicity. Multiplying the dose expressed in milligrams/kilograms by a factor of 7 yields a dose in milligrams/square meter that permits comparisons of toxicity between various species (11). On Day 7 after the start of the i.p. angiogenic treatment, the left jugular vein was opened in animals that had been anesthetized with inhaled isoflurane (Forene®, Abbott). One milliliter of the chemotherapeutic drug solution was injected into the jugular vein over a period of 1 to 2 min; controls received 1 ml physiological saline, 0.9% (w/v) NaCl. The skin incision was sutured immediately after drug injection. The animals were sacrificed on Day 14, which is safely within the expansive phase of the angiogenic reaction, which peaks around Day 21 (9). None of the animals receiving chemotherapy was lost.

Table 1

Elimination half-life and intravenous doses of the chemotherapeutic drugs used in the current study

Drug	Class*	Half-life in rats (h)	Doses i.v., mg/kg	Formulation
Fluorouracil (5-FU) Fluorouracil Faulding®, Baxter	Pyrimidine analogue	0.22–0.54[a]	10, 30, 50	NaOH q.s. ad pH 8.9 aqua ad inject. 1 ml
Paclitaxel Taxol®, Bristol-Myers Squibb	Taxane, a microtubule-targeting anticancer drug	7.6±3.6[b]	2, 6, 10	Ethanol 396 mg/ml, Cremophor® EL 527 mg/ml
Doxorubicin hydrochlorate Adriamycin®, Pharmacia	Intercalating topoisomerase-targeting drug	2.5±0.75[c]	4, 17, 30	0.75 mg/ml para-hydroxibenzoas, 37.5 mg/ml lactos[¶]
Cisplatin Platinol®, Bristol-Myers Squibb	Platinum antitumour compound	5–7[d], 0.15[e]	1, 3, 5	NaCl 9 mg/ml, HCl ad pH 2.5 aqua inject. ad 1 ml
Methotrexate Methotrexate®, Wyeth Lederle	Folate antagonist	0.4–0.6[f]	5, 20, 50	NaCl 4.9 mg/ml, NaOH aut HCl pH 8.5–8.9
Cyclophosphamide Sendoxan®, ASTA Medica	An alkylating agent	0.5–1.0[g]	30, 60, 120	57 mg/ml mannitol
Etoposide Etopofos®, Bristol-Myers Squibb	Non-intercalating topoisomerase-targeting drug	0.5–1.0[h]	2, 10, 20	1.6 mg/ml Na-citrate, 15 mg/ml Dextran 40

* According to the nomenclature in Holland et al. (eds.). Chemotherapeutic agents. Cancer Medicine 5th ed. 2000.
[a] (27), [b](28), [c](29), [d](30) for cisplatin and its active metabolites; [e](31) for unchanged cisplatin; [f](32); [g](33); [h](34). Cyclophosphamide is not a reactive compound but it undergoes activation in the body.
[¶] For the highest dose, otherwise physiologic saline. Mean±S.D.

Table 2

Effect of chemotherapy on body-weight gain

Dose i.v. (mg/kg)	Mean body weight in grams of saline-treated controls and of test groups, in %		
	Day 7	Day 10	Day 14
Fluorouracil			
Saline (12)	281.3	310.0	342.7
10 (10)	101%	101%	101%
30 (10)	99%	98%	99%
50 (10)	99%	94%	96%
Paclitaxel			
Saline (12)	276.8	305.6	344.7
2 (10)	98%	97%	97%
6 (10)	100%	97%	98%
10 (9)	99%	95%	96%
Doxorubicin			
Saline (8)	271.4	297.6	332.6
4 (10)	102%	97%	96%
17 (10)	100%	92%	89%
30 (14)	101%	88%	78%
Cisplatin			
Saline (12)	272.9	307.1	337.7
1 (10)	98%	97%	97%
3 (10)	99%	94%	91%
5 (10)	99%	93%	91%
Methotrexate			
Saline (12)	288.2	316.7	350.2
5 (10)	98%	96%	97%
20 (10)	99%	92%	92%
50 (10)	100%	89%	87%
Cyclophosphamide			
Saline (10)	269.9	295.7	330.1
30 (10)	103%	100%	101%
60 (10)	101%	96%	96%
120 (10)	103%	94%	90%
Etoposide			
Saline (11)	276.5	298.7	336.5
2 (10)	103%	102%	102%
10 (10)	102%	97%	97%
20 (10)	102%	92%	93%

Figures in parentheses indicate the number of animals. The animals received chemotherapy on Day 7 and were sacrificed on Day 14. Doxorubicin at 30 mg/kg reduced the body weight on Days 10 and 14, whereas methotrexate at 50 mg/kg and etoposide at 20 mg/kg reduced the body weight on Day 10 compared with the weight on Day 7.

Angiogenesis quantification

Four mesenteric windows were selected from the most distal part of the mesentery and spread on objective slides (9, 12). Normally, this tissue is sparsely vascularized, measures only 5–10 μm in thickness and forms a relatively uniform, almost translucent membrane ('window'). The surrounding fatty tissue distinctly delineates each window. The entire vasculature of each intact mesenteric window was visualized immunohistochemically (Fig. 1) using a primary monoclonal antibody to the rat endothelium (13).

Using microscopic morphometry and computerized digital image analysis in a blinded fashion, the total area of each window was measured and the microvasculature was objectively assessed in terms of the vascularized area (VA), a measurement of the spatial extension of the microvascular network, and microvascular length (MVL), a composite measurement of microvessel density (12). The total microvascular length (TMVL) was computed as the VA × the mean MVL of the treatment group. In addition, the following objective variables were measured within central parts of the expanding microvessel network (14, 15): the Le. MS (length of individual microvessel segments, i.e. the distance between two successive branching points), the median Le. MS, the No. MS (number of segments per unit tissue volume), the No. BP (number of branching points per unit tissue volume), the MVT (degree of tortuosity), the In. IS (index of intersection) and the In. LF (index of interconnecting loop formation). In each treatment group, the Le. MS was ranked in order of size. For statistical comparisons between the treatment groups, the 0–10 and 90–100 percentiles of the Le. MS were used.

Although some of the variables interrelate (14), it is fair to say that: a) the VA, MVL and TMVL are primarily measurements of microvessel proliferation; b) the No. MS and No. BP reflect both microvessel proliferation and pattern formation; c) the Le. MS measures the actual microvessel segment length; and d) the MVT, In. IS and In. LF primarily relate to microvessel pattern formation.

Statistics

The non-parametric Mann–Whitney U-test for unpaired (two-tailed) observations was used. The criterion for statistical significance was $p \leq 0.05$. A mean of four windows per animal was used as independent data for each variable.

RESULTS

Effect of chemotherapy on body-weight gain and test tissue size

No overall relationship was found between either drug half-life (Table 1) or body weight (Table 2) and the degree of antiangiogenic activity (Fig. 2, Tables 3 and 4), indicating that the angiogenesis-modulating effects were drug specific. The most prominent decrease in body-weight gain compared with controls and, in some cases, a decrease in body weight, occurred during the 3-day post-treatment period and was most pronounced following treatment with doxorubicin and methotrexate at the highest doses (Table 2). In the subsequent 4-day period, doxorubicin in the first place and also cisplatin, at intermediate and high doses, and methotrexate and cyclophosphamide, at the highest dose, had a marked impact on body-weight gain (Table 2).

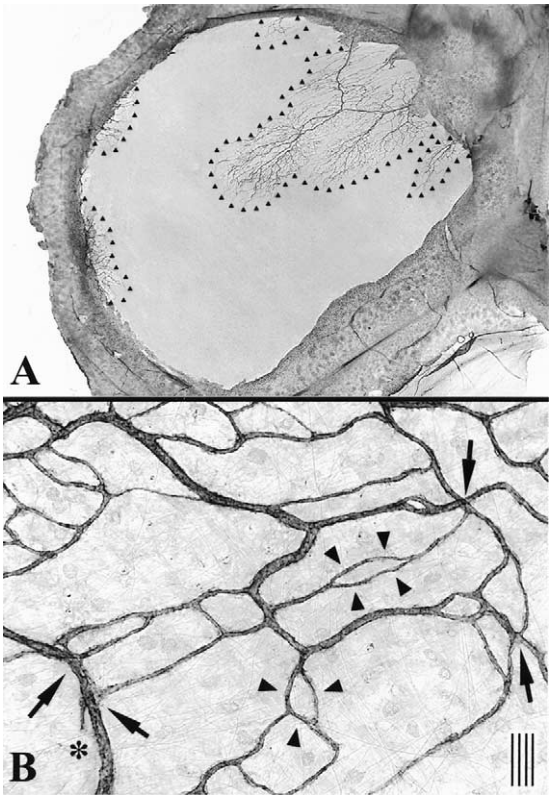

Fig. 1. Microvessel network visualized by specific histochemical staining of endothelial cells in a spread on an objective slide of intact control mesenteric window distinctly surrounded by fatty tissue. A. Overview. The 'window' area measures 1.2 cm^2. Arrowheads indicate vascularized areas at the periphery of the window. The sum of all vascularized areas as a percentage of the whole area, i.e. VA, was 27.8. B. Network details at higher magnification. Intersections are indicated by an arrow; the end of a sprout is indicated with an asterisk and arrowheads encircle the individual interconnecting loops (not all loops are marked). The distance between two bars is 10 μm.

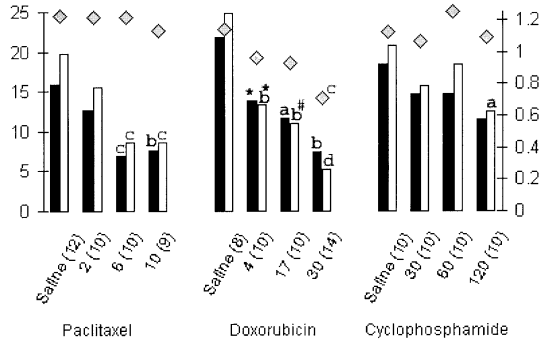

Fig. 2. Effects compared to saline controls in terms of vascularized area (VA, mean; left y-axis), microvascular length (MVL, mean; right y-axis) and total microvascular length (TMVL, mean; left y-axis) per animal. Figures in parentheses indicate the number of animals. a) $p \leq 0.05$; b) $p \leq 0.01$; c) $p \leq 0.005$ and d) $p \leq 0.0002$ compared with saline controls. *) $p \leq 0.02$ and #) $p \leq 0.01$ compared with doxorubicin 30 mg/kg. ■ VA; □ TMVL; ◇ MVL.

Microscopically, the test tissue had a normal appearance following treatment with all drugs at all doses. Mesenteric window size was unaffected by the chemotherapeutic treatment; the mean area of each control window was 1.07 cm^2 (detailed data not shown). As a result, all the treatment groups within each experiment can be compared with respect to the variables listed in Tables 3 and 4 and shown in Fig. 2.

Effects of chemotherapy on angiogenesis

Because the tables would be rather long if all the data were presented, only results that differ significantly in statistical terms from controls are included in Tables 3 and 4 and in Fig. 2.

Fluorouracil

Although not exerting a significant antiangiogenic effect, fluorouracil significantly affected some variables relating to the microvessel network pattern.

The low dose (10 mg/kg) significantly increased the index of microvessel interconnective loop formation (In. LF; Table 3). The difference in In. LF between the low- and high-dose (50 mg/kg) treatments was significant ($p \leq 0.015$). At the intermediate dose (30 mg/kg), the drug significantly lengthened the longest microvessel segments, i.e. increased the 90–100 percentile of the Le. MS. The intermediate dose also increased the median Le. MS by 11% (Table 4).

Paclitaxel

Paclitaxel strongly suppressed angiogenesis and affected several variables relating to the microvessel network formation.

At the intermediate and high doses (6 and 10 mg/kg), paclitaxel significantly reduced microvessel spatial extension in terms of the vascularized area (VA) by 56% and 52% and the overall angiogenic response in terms of the total microvascular length (TMVL) by 56% (Fig. 2). The difference in VA between treatments with the low and either the intermediate or the high dose was significant. Comparisons of the combined data for the intermediate and high doses with those of controls revealed statistically significant differences in VA and TMVL ($p \leq 0.005$ and $p \leq 0.0001$, respectively).

The MVT was significantly increased following treatment with the high dose of paclitaxel compared with controls (Table 3). Following treatment with the high dose, the In. IS was decreased by 30%, which was statistically significant (Table 3). At the high dose, the number of microvessel segments per unit tissue volume (No. MS) was significantly reduced compared either with controls or with animals treated with the intermediate dose (Table 4). The distribution of the Le. MS was virtually unaffected by paclitaxel treatment (Table 4 and Fig. 3).

Table 3

Statistically significant effects compared to saline controls in terms of number of microvessel branching points per unit tissue volume (No. BP), degree of microvessel tortuosity (MVT), index of interconnecting loop formation (In. LF) and index of microvessel intersection (In. IS) per animal (mean ± SEM). For all doses tested, see Table 1

Dose i.v. mg/kg	No. BP	MVT	In. LF	In. IS
Fluorouracil				
Saline (12)	214.9 ± 24.6	8.5 ± 0.3	1.137 ± 0.013	19.19 ± 1.13
10 (10)	311.6 ± 29.1*	8.4 ± 0.2	**1.200 ± 0.035**[a]	20.23 ± 1.23
Paclitaxel				
Saline (12)	263.7 ± 19.1	9.3 ± 0.3	1.144 ± 0.032	23.66 ± 1.35
10 (9)	217.6 ± 16.0*	**12.3 ± 1.6**[a]	1.115 ± 0.028	**16.61 ± 1.60**[a]
Doxorubicin				
Saline (8)	219.2 ± 23.9	9.2 ± 0.2	1.098 ± 0.013	15.78 ± 1.19
30 (14)	**120.3 ± 14.3**[c]	9.3 ± 0.3	1.063 ± 0.031	16.02 ± 1.84
Cisplatin				
Saline (12)	151.8 ± 15.7	8.0 ± 0.2	1.157 ± 0.030	14.06 ± 0.79
3 (10)	188.8 ± 26.7	9.1 ± 0.5	**1.098 ± 0.011**[a]	14.82 ± 2.02
Methotrexate				
Saline (12)	202.3 ± 24.0	7.8 ± 0.3	1.112 ± 0.018	18.25 ± 2.62
20 (10)	174.7 ± 29.2	8.3 ± 0.4	1.106 ± 0.018	**12.41 ± 1.54**[a]
Cyclophosphamide				
Saline (10)	261.0 ± 37.3	8.2 ± 0.8	1.161 ± 0.011	14.64 ± 1.04
30 (10)	206.4 ± 29.9	8.6 ± 0.6	**1.104 ± 0.013**[b]	15.61 ± 1.81
Etoposide				
Saline (11)	213.2 ± 19.2	9.2 ± 0.3	1.124 ± 0.012	14.80 ± 1.83
20 (10)	205.9 ± 25.9	9.6 ± 0.3	**1.086 ± 0.014**[a]	18.58 ± 2.08

Figures in parentheses indicate the number of animals. Figures in bold type indicate a significant difference compared with saline controls. * $p \leq 0.10$; [a]$p \leq 0.05$; [b]$p \leq 0.01$; [c]$p \leq 0.005$ compared with saline controls.

Doxorubicin

Angiogenesis was significantly suppressed by doxorubicin at all three doses. The microvessel network pattern was also significantly affected in some aspects.

Intermediate and high (17 and 30 mg/kg, respectively) doses of doxorubicin significantly subdued the VA (by 46% and 66%, respectively) compared with controls (Fig. 2). The high dose also significantly reduced the MVL (Fig. 2). In addition, all three doses of the drug reduced the TMVL significantly (Fig. 2). In terms of TMVL, the difference between either the low (4 mg/kg) or the intermediate dose and the high dose was significant.

The No. BP and the No. MS decreased significantly at the high dose (Tables 3 and 4). Moreover, all doses of doxorubicin significantly lengthened the Le. MS within the 90–100 percentile and increased the median Le. MS by 17–18% (Table 4 and Fig. 3).

Cisplatin

Treatment with cisplatin had no significant antiangiogenic effect. However, some variables relating to the microvessel network formation were significantly affected.

Compared with controls, the intermediate dose (3 mg/kg) significantly reduced the In. LF (Table 3). At all three doses, cisplatin significantly affected the Le. MS, in that both the 0–10 percentile and the 90–100 percentile were significantly elongated. The low (1 mg/kg), intermediate and high (5 mg/kg) doses increased the median Le. MS by 10%, 19% and 17%, respectively (Table 4).

Methotrexate

Although not exerting a significant antiangiogenic effect, methotrexate significantly affected several variables relating to the microvessel network pattern.

The intermediate dose (20 mg/kg) of methotrexate reduced the In. IS by 32%, which was statistically significant compared with the control (Table 3). The intermediate dose was also efficacious in affecting the Le. MS (Table 4): both the 0–10 and the 90–100 percentiles of the Le. MS were increased significantly compared with controls. The low dose (5 mg/kg) significantly lengthened the Le. MS within the 90–100 percentile. The differences in terms of the 0–10 Le. MS percentile between the intermediate dose and either the low or the high dose were significant ($p \leq 0.02$ and $p \leq 0.005$, respectively). The difference between the intermediate and high doses in terms of the Le. MS 90–100 percentile was also significant ($p \leq 0.01$). The low and intermediate doses increased the median Le. MS by 13% and 14%, whereas the high dose increased the median Le. MS by only 4% (Table 4).

Table 4

Statistically significant effects compared with those of saline controls in terms of number of microvessel segments per unit tissue volume (No. MS) per animal (mean ± SEM), median length of microvessel segments (Le. MS) and shortest and longest Le. MS, i.e. the (0–10) and the (90–100) percentiles, for the whole population of Le. MS in each treatment group. For all doses tested, see Table 1

Dose i.v. mg/kg	No. MS	Le. MS[§]		Le. MS median, μm
		0–10 percentile	90–100 percentile	
Fluorouracil				
Saline (12)	295.7 ± 36.2			69.2 (100%)
30 (10)	286.7 ± 21.1	n.s.	**$p \leq 0.0025$, /**	76.8 (111%)
Paclitaxel				
Saline (12)	305.7 ± 27.4			66.6 (100%)
10 (9)	**241.7 ± 17.6**[a]	n.s.	n.s.	69.2 (104%)
Doxorubicin				
Saline (8)	242.7 ± 28.8			66.3 (100%)
4 (10)	168.7 ± 29.6[*]	n.s.	**$p \leq 0.0001$, /**	78.1 (118%)
17 (10)	178.0 ± 17.6[*]	n.s.	**$p \leq 0.0001$, /**	78.1 (118%)
30 (14)	**129.9 ± 16.9**[b]	n.s.	**$p \leq 0.003$, /**	77.4 (117%)
Cisplatin				
Saline (12)	173.6 ± 17.7			60.2 (100%)
1 (10)	192.0 ± 23.2	**$p \leq 0.02$**, /	**$p \leq 0.01$, /**	66.6 (110%)
3 (10)	205.7 ± 33.5	**$p \leq 0.0001$, /**	**$p \leq 0.001$, /**	71.7 (119%)
5 (10)	215.8 ± 32.6	**$p \leq 0.0001$, /**	**$p \leq 0.05$, /**	70.4 (117%)
Methotrexate				
Saline (12)	227.7 ± 29.3			61.5 (100%)
5 (10)	219.3 ± 20.7	n.s.	**$p \leq 0.002$, /**	69.2 (113%)
20 (10)	197.2 ± 36.1	**$p \leq 0.0001$, /**	**$p \leq 0.0001$, /**	70.4 (114%)
50 (10)	255.3 ± 29.8	n.s.	n.s.	64.0 (104%)
Cyclophosphamide				
Saline (10)	306.4 ± 45.8			62.4 (100%)
30 (10)	230.2 ± 34.6	n.s.	**$p \leq 0.0001$, /**	74.5 (119%)
60 (10)	293.5 ± 21.7	**$p \leq 0.005$, /**	**$p \leq 0.0001$, /**	68.9 (110%)
120 (10)	255.3 ± 32.5	**$p \leq 0.002$, /**	**$p \leq 0.05$, /**	68.3 (109%)
Etoposide				
Saline (11)	241.0 ± 23.4			60.3 (100%)
2 (10)	256.0 ± 20.7	**$p \leq 0.0005$, /**	**$p \leq 0.003$, /**	72.7 (121%)
10 (10)	248.8 ± 24.4	**$p \leq 0.0001$, /**	**$p \leq 0.003$, /**	68.9 (114%)
20 (10)	226.8 ± 31.2	n.s.	**$p \leq 0.0003$, /**	68.8 (114%)

Figures in parentheses indicate the number of animals. Figures in bold type indicate a significant difference compared with saline controls. P-values show differences compared with controls.
[§] compared with Le. MS 0–10 and 90–100 percentiles of controls. Elongation of Le. MS, /. Not significant in statistical terms, *n.s.*
* $p \leq 0.10$; [a] $p \leq 0.05$; [b] $p \leq 0.005$ compared with controls.
The distribution of Le. MS—i.e. the 0–10 percentile, the 90–100 percentile and the median Le. MS—was based on populations of between 3 548 (saline control in the paclitaxel experiment) and 1 819 (doxorubicin 30 mg/kg) individually measured Le. MS.

Cyclophosphamide

At the high dose, cyclophosphamide significantly suppressed angiogenesis. The treatment with cyclophosphamide also affected some network pattern variables.

The high dose of cyclophosphamide significantly reduced the TMVL ($p \leq 0.05$, Fig. 2). At the low dose (30 mg/kg), the In. LF was significantly reduced ($p \leq 0.01$, Table 3). The low dose also lengthened the 90–100 percentile of the Le. MS to a highly significant degree and increased the median Le. MS by 19% (Table 4). The intermediate (60 mg/kg) and high doses significantly lengthened both the 0–10 and the 90–100 percentiles of the Le. MS, and the median Le. MS was increased by 10% and 9%. Compared with controls, the low and intermediate doses affected the longest Le. MS to a greater extent ($p \leq 0.0001$) than was the case with the high dose ($p \leq 0.05$; Table 4).

Etoposide

Although not exerting any significant antiangiogenic effect, treatment with etoposide affected some microvessel network pattern variables.

The high dose (20 mg/kg) of etoposide significantly reduced the In. LF (Table 3). At all doses, etoposide significantly lengthened the Le. MS, i.e. the median Le.

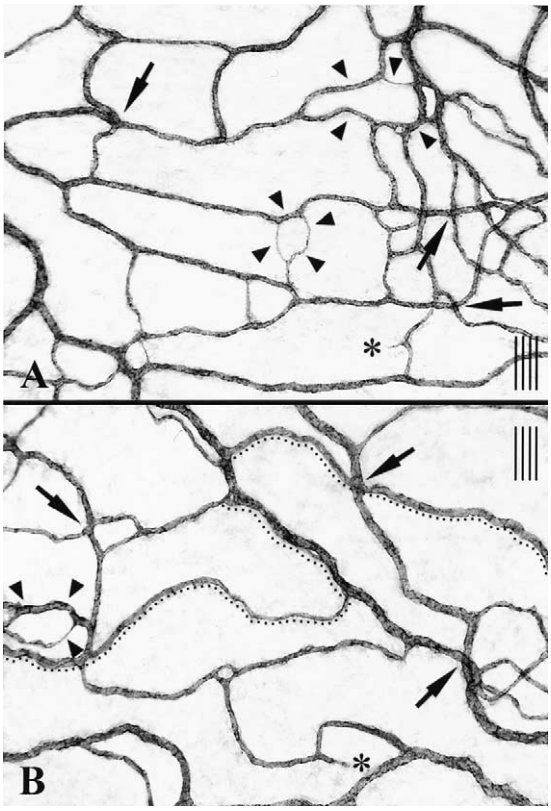

Fig. 3. Microvessel network details following treatment with paclitaxel at 6 mg/kg (A) and doxorubicin at 4 mg/kg (B). In (A), notice the overall resemblance to the network in Fig. 1B. In (B), notice the occurrence of two very long microvessel segments (indicated by a dotted line). Both these long segments are incomplete, however, as the full distance between the two successive branching points is not included in the Figure. Note that one of the extra long microvessel segments runs in part close to a somewhat wider microvessel. Intersections are indicated by an arrow; the end of a sprout is indicated by an asterisk and arrowheads encircle individual interconnecting loops (not all loops are marked). The distance between two bars is 10 μm.

MS was increased by 21% by the low dose (2 mg/kg) and 14% by both the intermediate (10 mg/kg) and high doses. All doses significantly lengthened the Le. MS within the 90–100 percentile, whereas the low and intermediate doses also lengthened the 0–10 percentile of the Le. MS (Table 4).

Both the controls and the test animals experienced a moderate anaphylactoid reaction to the vehicle, which contained dextran.

COMMENTS

As adult rats grow considerably faster than do adult mice (16), the effect of chemotherapy on body growth should be a considerably more sensitive indicator of general toxic effects (including systemic well-being, anorexia, and failure to thrive) in rats compared with mice. This might be helpful in precluding any confusion about the toxicity and anti-angiogenic efficacy of drugs. Obviously, the effects of chemotherapy on body weight reported here cannot be directly compared with those seen in studies involving mice.

Depending on the drug and dose that were used, chemotherapy induced significant decreases in the VA, MVL, TMVL, No. BP, No. MS and In. IS (Fig. 2; Tables 3 and 4). The In. IS is also reduced by the oral administration of iron-unsaturated bovine lactoferrin which inhibits $VEGF_{165}$-mediated angiogenesis in the rat mesenteric-window angiogenesis assay (13). However, the MVT increased significantly at the high dose of paclitaxel (Table 3) and an increased level of MVT has been found following low-molecular-weight heparin treatment that inhibits $VEGF_{165}$-induced angiogenesis (17). The In. LF was significantly increased by the low dose of fluorouracil (Table 3). In contrast, the intermediate dose of cisplatin, the low dose of cyclophosphamide and the high dose of etoposide significantly reduced In. LF (Table 3). None of the drugs exerted any obvious pro-angiogenic effects.

Our results suggest that the elongation of the Le. MS is commonly associated with chemotherapy and is not necessarily related to the impairment of microvessel branching and interconnecting loop formation. The Le. MS is, in fact, influenced by many factors, including the ability of the microvessels to elongate, branch and form interconnecting loops. An inverse relationship between the No. MS, No. BP and In. LF, on the one hand, and the median Le. MS or the 90–100 percentile Le. MS, on the other, can therefore be expected, as was observed in animals treated with doxorubicin, cisplatin, cyclophosphamide or etoposide (Tables 3 and 4). Furthermore, intermediate doses of fluorouracil barely affected the No. MS, No. BP or In. LF. In contrast, the median Le. MS increased by 11% and the 90–100 percentile of the Le. MS was significantly lengthened by fluorouracil in comparison with controls.

The dose-response curve for the Le. MS was bell-shaped in many of the experiments, as the intermediate doses displayed the highest efficacy in elongating the Le. MS (Table 4). Paclitaxel elicited a unique dose-independent response pattern in that it did not affect the Le. MS distribution but the high dose reduced the No. MS (Table 4). Although the significance of the Le. MS lengthening remains to be elucidated, it is noteworthy that the Le. MS, in contrast, is initially shortened in $VEGF_{165}$-induced angiogenesis (14).

Only doxorubicin produced an approximately linear dose-dependent antiangiogenic effect (Fig. 2). Despite the fact that doxorubicin inhibited angiogenesis effectively and lengthened the Le. MS, it did not alter the MVT, In. IS or In. LF (Table 3). Significant effects on the MVT, In. IS or In. LF, corresponding to changes in microvessel pattern formation, were found following treatment with all the drugs except doxorubicin (Table 3).

DISCUSSION

Miller et al. (4) recently reviewed the literature on the issue of antiangiogenic activity of chemotherapeutics in vitro and found that most agents tested (a multitude of concentrations have been used), including paclitaxel, cyclophosphamide and doxorubicin, suppress 'angiogenesis' in terms of endothelial cell proliferation, migration and tubule formation. On the other hand, agents such as cisplatin and fluorouracil have demonstrated antiangiogenic activity only in one or two of these in vitro assays. Relatively few chemotherapeutics have been shown to affect angiogenesis in vivo, however (4). The data presented here are the first evidence of a dose-response effect on VEGF-induced angiogenesis, or any other type of angiogenesis for that matter, in vivo for a panel of representative examples of major chemotherapeutic agents. The agents represent seven different classes. From a clinical perspective, candidate cytotoxics should ideally exert a 'type-1' reaction pattern; i.e. the agent should act directly on the microvessel endothelial cells (18, 19). Thus the present results indirectly mirror the effects of 'type-1' antiangiogenic chemotherapeutics on tumors, since tumor angiogenesis apparently uses the same signaling pathways as non-tumor angiogenesis (20).

The effect of metronomic chemotherapy treatment of both ectopic s.c. and orthotopic breast tumors is, reportedly, to a great extent or even primarily antiangiogenic in inbred C57Bl6/J and CB-17 SCID mice (1, 2, 21). With the current methodology, the exact contribution of the antiangiogenic effect on the recorded anti-tumor effect eludes elucidation, however. Most importantly, established drug-resistant tumors showed complete regression following combined metronomic chemotherapeutic and antiangiogenic therapy (TNP-470 or monoclonal neutralizing antibody targeting the type 2 receptor for VEGF). This strategy of combined treatment with one or more chemotherapeutic drugs administered metronomically and one or more antiangiogenic agents could be the future therapeutic anticancer strategy of choice, especially as it minimizes or delays problems with host toxicity and acquired drug resistance (22, 23).

Even in cases where mouse tumors had been pre-selected for acquired resistance to a chemotherapeutic drug (1, 2), the influence of chemotherapy on tumor angiogenesis is tumor dependent, as tumors are heterogeneous and unique. Moreover, the influence of chemotherapy on tumor angiogenesis may be tissue and site dependent (18, 24, 25). This makes it difficult to draw conclusions about any generally convincing force from one specific tumor or tumor model. The superior antitumor effect by metronomic as compared with conventional chemotherapy treatment, as reported by Browder et al. (1) and Klement et al. (2, 21), is probably that the endothelial cells recover during the 3-week rest period and restore the blood supply to the tumor. Owing to endothelial cell heterogeneity and tissue-specific properties, the inhibition of angiogenesis in s.c. neoplasms, s.c. Matrigel plugs and corneas (1, 2) may not predict the inhibition of angiogenesis in metastatic visceral organs, such as the lungs, liver, bone and brain. Notably, visceral metastases usually result in death. The use of a visceral tissue in the present study would make the results highly relevant to tumors growing in the peritoneum and possibly to visceral cancers in general.

In order to address the antiangiogenic effect of chemotherapeutics *per se*, we studied the effect of cytotoxics on angiogenesis in a tumor-free model, although this model also has limitations. For example: a) the influence of chemotherapy on angiogenesis in normal tissue is probably normal-tissue dependent and perhaps even site (mesentery) dependent; b) the effect of chemotherapeutics on tumor vascular channel formation (vasculogenic mimicry), which is independent of angiogenesis but might play a role in tumor growth (26), is not accessible; c) any influence of chemotherapy on endothelial cell apoptosis that is followed by tumor cell apoptosis after chemotherapy (1) defies assessment; d) in tumors, multiple pathways may often induce angiogenesis, at least in tumors showing advanced progression, which eludes analysis in the present study. However, apart from being a major angiogenic factor in its own right in most tumors, VEGF is also known to play a role in angiogenic pathways that are related to other angiogenic factors (7).

Although there was no observable difference in the present study in the response of the mesenteric vasculature to the one dose of fluorouracil, cisplatin, methotrexate, or etoposide, a single dose of paclitaxel or doxorubicin exerted significant antiangiogenic effects. Cyclophosphamide fell in the moderate range, exhibiting an intermediate effect on the vasculature. These results suggest that there are significant variations in antiangiogenic effects among standard chemotherapeutic drugs. Although paclitaxel had a marginal effect on body-weight change, at the intermediate and high doses, it exerted the strongest antiangiogenic effect, reducing both the VA and TMVL to a highly significant degree. Similarly, low doses of doxorubicin did not affect body weight to any marked degree but reduced the TMVL significantly. At higher doses, doxorubicin suppressed angiogenesis effectively but also caused body-weight loss or a substantial retardation in body-weight gain (Table 2). The high dose of cyclophosphamide, which was antiangiogenic, lowered body weight by 10% by the day of sacrifice.

The pharmacokinetics (such as half-life) and pharmacodynamics of each drug that was used influenced the antiangiogenic effects investigated here. Paclitaxel and doxorubicin exerted significant antiangiogenic effects and doxorubicin produced a linear-like dose response (Fig. 2). It is possible that the doses selected for the other drugs were too low. Nevertheless, although methotrexate at the high

dose caused a relatively strong growth-retarding effect (Table 2), it did not exert a significant overall antiangiogenic effect (Fig. 2; Tables 3 and 4). It is noteworthy that both paclitaxel and doxorubicin have longer half-lives than 5-fluorouracil, methotrexate and etoposide (Table 1). However, cisplatin and its active metabolites also have long half-lives but still cisplatin did not exert any significant antiangiogenic effect, although it did reduce the In. LF and lengthened the Le. MS. A dosage regimen other than the single bolus schedule that was used here might show a more authentic antiangiogenic effect owing to sustained plasma and tissue concentrations of the drugs. Constant drug infusion, which represents the extreme of the metronomic treatment modality, will reveal the combined influence of the pharmacokinetic and pharmacodynamic factors for each drug and thus further elucidate the true antiangiogenic potential.

In on-going studies we will determine whether the antiangiogenic response when assessed 7 days after the drug bolus exposure, as in the present study, may have underestimated the effectiveness of lower doses compared with the antiangiogenic effect of lower doses administered as constant infusion using s.c. osmotic pumps. Furthermore, the effectiveness of the combined treatment with one or more antiangiogenic agents with continuously infused chemotherapeutics at low doses will be assessed. Studies of rat tumor models using agents and doses that were antiangiogenic in the present study are in progress.

Regarding the possible clinical implications of the present results, it interesting that in patients with metastatic breast cancer a substantial clinical response rate (38%) with minimal toxicity has been seen after a low-dose schedule including cyclophosphamide and methotrexate (35). It is, moreover, noteworthy that the non-responders in that study showed increased serum VEGF levels. Furthermore, and in accordance with the preclinical data presented here, several weekly taxan schedules demonstrate a favorable effect-toxicity profile, and an antiangiogenic drug action is at least to some extent thought to cause the favorable effects (36).

We agree with others (2, 3, 19) that it is necessary to assess the most potent antiangiogenic chemotherapeutic agents in relevant models, at different dosages and with different treatment schedules in terms of their intrinsic antiangiogenic and antitumor effects. That each preclinical model has limitations goes without saying. Moreover, the long-term toxicity testing of drugs at dosages and treatment schedules that act antiangiogenically is probably needed in each of the species concerned in order to check for late-appearing, unforeseeable, accumulating serious toxic effects. We believe that the rat mesentery assay may represent a useful supportive complement to various established mouse and rat tumor models in these types of study as it permits the objective, quantitative assessment of dose-related anti-angiogenic effects *per se*.

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REFERENCES

1. Browder T, Butterfield CE, Kräling BM, Shi B, Marshall B, O'Reilly MS, Folkman J. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 2000; 60: 1878–86.
2. Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest 2000; 105: R15–24.
3. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 2000; 105: 1045–7.
4. Miller KD, Sweeney CJ, Sledge Jr GW. Redefining the target: chemotherapeutics as antiangiogenics. J Clin Oncol 2001; 19: 1195–206.
5. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000; 407: 249–57.
6. Stein I, Keshet E. Regulation of vascular endothelial growth factor (VEGF) expression. In: Voest EE, D'Amore PA, eds. Tumor angiogenesis and microcirculation. New York: Marcel Dekker, 2001: 213–26.
7. Ferrara N. Role of vascular endothelial growth factor in angiogenesis. In: Rubanyi GM, ed. Angiogenesis in health and disease. New York: Marcel Dekker, 2001: 47–73.
8. Workman P, Twentyman P, Balkwill F, et al. United Kingdom co-ordinating committee on cancer research (UKCCCR) guidelines for welfare of animals in experimental neoplasia. 2nd ed. Br J Cancer 1998; 77: 1–10.
9. Norrby K. Vascular endothelial growth factor and de novo mammalian angiogenesis. Microvasc Res 1996; 51: 154–63.
10. Mukhopadhyay D, Nagy JA, Manseau EJ, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor-mediated signaling in mouse mesentery vascular endothelium. Cancer Res 1998; 58: 1278–84.
11. Freireich EJ, Gehan EA, Rall DP, Schmidt LH, Skipper HE. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother Rep 1966; 50: 219–44.
12. Norrby K. Basic fibroblast growth factor and de novo mammalian angiogenesis. Microvasc Res 1994; 48: 96–113.
13. Norrby K, Mattsby-Baltzer I, Innocenti M, Tuneberg S. Orally administered bovine lactoferrin systemically inhibits VEGF165-mediated angiogenesis in the rat. Int J Cancer 2001; 91: 236–40.
14. Norrby K. Microvascular density in terms of number and length of microvessel segments per unit tissue volume in mammalian angiogenesis. Microvasc Res 1998; 55: 43–53.
15. Näslund I, Norrby K. NO and de novo angiogenesis: Further evidence that NO inhibits bFGF-induced angiogenesis while not influencing VEGF165-induced angiogenesis. APMIS 2000; 108: 29–37.
16. Poiley SM. Growth tables for 66 strains and stocks of laboratory animals. Lab Animal Sci 1972; 22: 759–79.

17. Norrby K. 2.5 kDa and 5.0 kDa heparin fragments specifically inhibit microvessel sprouting and network formation in VEGF165-mediated mammalian angiogenesis. Int J Exp Path 2000; 81: 191–8.
18. Kerbel RS. Tumor angiogenesis: past, present and the near future. Carcinogen 2000; 21: 505–15.
19. Schirner M. Antiangiogenic chemotherapeutic agents. Cancer Metastasis Rev 2000; 19: 67–73.
20. StCroix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. Science 2000; 289: 1197–202.
21. Klement G, Huang P, Mayer B, et al. Differences in therapeutic indexes of combination metronomic chemotherapy and an anti-VEGFR-2 antibody in multi-resistant human breast cancer xenografts. Clin Cancer Res 2002; 8: 221–32.
22. Kerbel RS, Viloria-Petit A, Klement G, Rak J. 'Accidental' anti-angiogenic drugs, anti-oncogene directed signal transduction inhibitors and conventional chemotherapeutic agents as examples. Eur J Cancer 2000; 36: 1248–57.
23. Folkman J, Browder T, Palmblad J. Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost 2001; 86: 23–33.
24. Fidler IJ, Ellis LM. Chemotherapeutic drugs — more really is not better. Natl Med 2000; 6: 500–2.
25. Ruoslahti E, Rajotte D. An address in the vasculature of normal tissues and tumors. Ann Rev Immunol 2000; 18: 813–27.
26. Folberg R, Hendrix MJ, Maniotis AJ. Vasculogenic mimicry and tumor angiogenesis. Am J Pathol 2000; 156: 361–81.
27. Venkateswar RJ, Lam SS, Boudinot FD. Nonlinear pharmacokinetics of 5-fluorouracil in rats. J Pharm Sci 1997; 86: 756–8.
28. Coudore F, Authier N, Guillaume D, Beal D, Duroux E, Fialip J. High-performance liquid chromatographic determination of paclitaxel in rat serum: application to a toxicokinetic study. J Chromatogr B Biomed Sci Appl 1999; 721: 317–20.
29. Gulyaev AE, Gelperina SE, Skidan IN, Antropov AS, Kivman GY, Kreuter J. Significant transport of doxorubicin into the brain with polysorbate 80-coated nanoparticles. Pharm Res 1999; 16: 1564–9.
30. Takada K, Kawamura T, Inai M, et al. Pharmacokinetics of cisplatin in analbuminemic rats. Biopharm Drug Dispos 1999; 20: 421–8.
31. Hanada K, Ninoiya K, Ogata H. Pharmacokinetics and toxicodynamics of cisplatin and its metabolites in rats: relationship between renal handling and nephrotoxicity of cisplatin. J Pharm Pharmacol 2000; 52: 1345–53.
32. Kim MM, Lee SH, Lee MG, Hwang SJ, Kim CK. Pharmacokinetics of methotrexate after intravenous and intramuscular injection of methotrexate-bearing positively charged liposomes to rats. Biopharm Drug Dispos 1995; 16: 279–93.
33. Yu LJ, Drewes P, Gustavsson K, Brain EG, Hecht JE, Waxman DJ. In vivo modulation of alternative pathways of P-450-catalyzed cyclophosphamide metabolism: impact on pharmacokinetics and antitumour activity. J Pharmacol Exp Ther 1999; 288: 928–37.
34. Burgio DE, Gosland MP, McNamara PJ. Effects of P-glycoprotein modulators of etoposide elimination and central nervous system distribution. J Pharmacol Exp Ther 1998; 287: 911–7.
35. Rocca A, Colleoni M, Nole F, et al. Low dose oral methotrexate and cyclophosphamide in metastatic breast cancer. Antitumor activity and correlations with serum vascular endothelium growth factor. Proc Am Soc Clin Oncol 1999; 18: 121a.
36. Marchetti P, Urien S, Cappellini GA, Ronzino G, Ficorella C. Weekly administration of paclitaxel: theoretical and clinical basis. Crit Rev Oncol Hematol (Suppl) 2002; 44: S3–13.