

Vascular density profile of rat mammary carcinomas induced by 1-methyl-1-nitrosourea: implications for the investigation of angiogenesis

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Given the mounting interest in studying the effects of various cancer preventive regimes on the angiogenic process in autochthonously growing mammary tumors, the experiment reported here was conducted to identify factors that should be considered in the collection of data from experiments in which a primary endpoint is the measurement of blood vessel density as a reflection of angiogenic activity. Female Sprague–Dawley rats were injected i.p. with 50 mg 1-methyl-1-nitrosourea (MNU)/kg body weight at 21 days of age. Tumor-bearing rats were killed 35 days post-carcinogen. Immunohistochemical detection of blood vessels using antiserum directed against the CD31 epitope was performed on paraffin sections of 36 carcinomas representing four histological types. Census counting was used to detect all blood vessels that were subsequently divided into one of five vessel size categories. Vessels were counted in both the intra-tumoral region and in a 50 µm width band of mammary tissue circumscribing each tumor; the circumscribed area was referred to as the extra-tumoral region. Vascular density was reported in units of vessel counts or vessel area per unit of assessed area. Overall, vascular density was observed to be highly variable and was not affected by tumor histology. Vascular density in the extra-tumoral region was significantly higher than in the intra-tumoral region. Correlation analyses indicated that similar information was obtained when vascular density was reported as either vessel counts or vessel area; however, correlations across vessel size categories and between the intra-tumoral and extra-tumoral regions were low and generally not statistically significant. Collectively, the data provide a complete description of vascularization in autochthonously growing mammary tumors, and a reference for comparison in studies of various cancer preventive agents that modulate the angiogenic process.

Introduction

Establishment of a new vascular supply, a process referred to as angiogenesis, is a critical factor that limits tumor growth (1,2). In the mid 1970s, Gullino *et al.* (3–5) demonstrated that angiogenesis was induced during chemically induced mammary carcinogenesis in the rat. However, the approaches used in those studies to assess angiogenesis were relatively crude and required secondary systems for quantification, specifically, the rabbit iris bioassay. Following those initial reports, only a

Abbreviations: APES, 3-aminopropyltriethoxysilane; MANOVA, multivariate analysis of variance; MNU, 1-methyl-1-nitrosourea.

limited amount of work has been directed towards investigating the development of a blood supply in experimentally induced autochthonous mammary tumor models that are widely used to study the cancer preventive activity of many agents (6). One of the issues confronted by investigators seeking to study this process *in vivo*, is whether to apply the same techniques currently used to study angiogenesis clinically. What becomes apparent when the relevant literature is reviewed (7) is that most clinical approaches are governed by time constraints related to pathology reporting, which introduce limitations that may be unnecessary and unwanted in the laboratory research setting. For example, it is common to identify tumor blood vessels by immunohistochemical staining and then to focus on quantifying blood vessel counts as a reflection of angiogenesis in highly stained regions of a tumor, the so called ‘hot spot’ technique (7). While this approach, and a variation of it referred to as the Chalkley technique, yield useful clinical information, they have a significant subjective component, i.e. the selection of areas that are counted for vessels using methods that are intentionally biased towards evaluating only highly vascularized regions. This is problematic in that effects on ‘hot spot’ areas may not accurately reflect the overall angiogenic state of a tumor or of the tissue in immediate proximity to a tumor. Moreover, molecular studies of mechanisms underlying the angiogenic process frequently derive tissue for analysis from areas other than those in which ‘hot spots’ are located. If such data are then related to angiogenic activity determined by the ‘hot spot’ technique, difficulties in data interpretation may arise. In addition, most approaches for studying tumor angiogenesis fail to consider new blood vessel formation in the tissue that is proximal to the tumor, i.e. extra-tumoral tissue. Consequently, it is not known with certainty which region, i.e. intra-tumoral or extra-tumoral, is more highly vascularized, and whether one or both regions should be assessed to determine the effects of agents that modulate the angiogenic process. This situation is further complicated by the complete lack of quantitative information about the density of blood vessels of different sizes within and around a carcinoma and about the variability of vascularization among mammary tumors of different histological types. Such information is particularly important in the design of experiments. The study reported here was conducted to provide a framework for deciding what data should be collected in cancer prevention experiments in which mammary carcinomas are chemically induced.

Materials and methods

The experiment reported in this study used a rapid emergence, chemically induced mammary carcinogenesis model (8) that is a modification of the procedure originally published by Gullino *et al.* (9). This model provides for the rapid screening of agents and dietary treatments for cancer preventive activity since a study can be completed 35 days from its inception. Moreover, given the short latency for tumor occurrence, the potential confounding effects of ‘tumor aging’ on vascularization are minimized. In addition, carcinomas appearing in the rapid emergence model are generally of smaller size compared

with those in the conventional 1-methyl-1-nitrosourea (MNU) model. The smaller tumor size enabled the process of image capture and census counting to be more manageable than it would have been otherwise. Furthermore, the short latency for tumor occurrence resulted in the formation of discrete tumors with ample surrounding stromal tissue, thus avoiding development of conjoined tumors, which can be problematic when trying to assess both intra- and extra-tumoral vascularization. Use of this model permitted a vigorous assessment of the variation inherent in the vascularization of carcinomas of different histological types all of which reached a detectable size within a period of 14 days or less.

Twenty-day-old female Sprague–Dawley rats were obtained from Taconic Farms, Germantown, NY. Rats were injected i.p. with 50 mg MNU/kg body weight at 21 days of age (10). Tumor-bearing rats were killed 35 days post-carcinogen. Whole mounts of abdominal inguinal mammary glands were prepared and fixed in 10% neutral buffered formalin for a period of 18–24 h, which has been reported to be the optimal length of fixation for immunostains (11,12). Whole mounts were stained with alum carmine. Lesions were identified, excised and processed for routine histological evaluation as described previously (8). The methods for immunostaining of mammary carcinomas have been reported in detail (13). Briefly, paraffin sections from formalin-fixed mammary carcinomas were cut at 4 μm and mounted onto 3-aminopropyltriethoxysilane (APES)-treated slides. Sections were then processed for immunostaining of blood vessels using antiserum directed against the CD31 epitope as recommended (13). Census counting of all blood vessels was undertaken both in the intra-tumoral region and in a 50 μm width band of mammary tissue circumscribing each tumor; the circumscribed area is referred to as the extra-tumoral region. Vessels were counted using images of immunostained sections captured with a Kodak DCS-420 digital camera (Eastman Kodak, Rochester, NY) mounted on a Zeiss Axioskop microscope using a 10 \times objective. The CCD imaging sensor on the DCS-420 has a focal magnification of 2.5 \times yielding images with an approximate total magnification of 250 \times . The resolution of each image was 1012 \times 1524 or 1.5 megapixels. Images were acquired using a 32 bit Kodak TWAIN driver v5.02 within Adobe Photoshop v4.0 graphic software (Adobe Systems, Inc., San Jose, CA) running on a 300 MHz Pentium III PC with 128 Mbyte of RAM. Lesions that exceeded the size of a single imaging area were captured by photographing contiguous microscopic fields in a raster pattern. Each captured image was merged using a layer technique in Adobe Photoshop to form a single composite image for analysis. All vessels were circumscribed manually using a digitizing pen to rule out inclusion of artefact or background immunostain. Criteria established by Weidner and Folkman were used to identify blood vessels in immunostained sections (14). Specifically, positively stained endothelial cells or endothelial cell clusters, regardless of size or shape, and that were clearly separate from adjacent blood vessels, tumor cells or other connective tissue elements were counted. Vessel lumens, although usually present, were not necessary for a structure to be defined as a blood vessel, and red blood cells were not used to define a vessel lumen. All data were exported to Systat v10.01 (SPSS, Chicago, IL) for statistical analyses. The specific statistical tests used are described in the Results section and table legends.

Results

As illustrated in Figure 1A, blood vessels are observed adjacent to epithelial structures in the normal mammary gland; whereas, they are found not only adjacent to mammary carcinomas (termed extra-tumoral blood vessels) but also within the carcinomas (intra-tumoral vessels) as shown in Figure 1B. The number and size of all blood vessels in both the intra-tumoral and extra-tumoral regions were quantified in 36 mammary carcinomas excised from 32 animals. Based on information gathered from the literature detailing the typical sizes of capillaries, venules and arterioles observed in the rat, and the average size of a red blood cell observed in formalin-fixed, paraffin embedded tissue, it was decided to divide blood vessels into five size categories as shown in Table I; the percentage of all detected blood vessels that were classified into each category also is listed. Vascular density values were then computed and expressed as number of vessels per unit of assessed area and as vessel area per unit of assessed area for each vessel size category.

Vessel density data for all carcinomas, irrespective of histological type, are summarized in Table II. Variability in

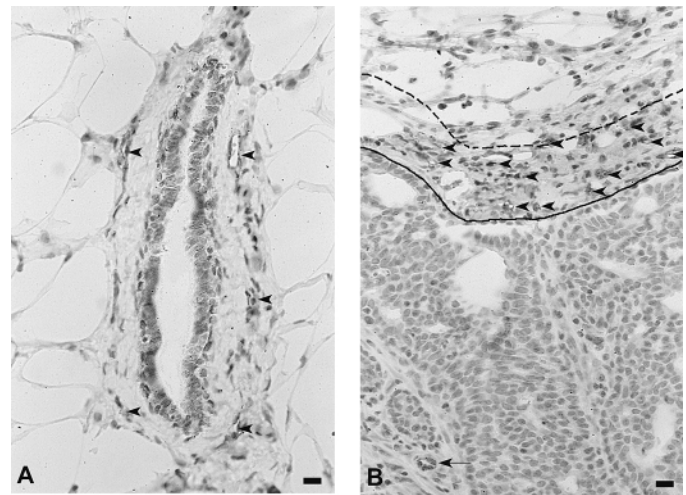


Fig. 1. (A) High magnification (400 \times) of normal rat mammary duct demonstrating CD31 stained blood vessels (arrowheads). (B) High magnification (400 \times) demonstrating CD31 stained blood vessels in a portion of rat mammary carcinoma. A solid black line delineates circumscribed tumor epithelium whereas the dashed line depicts the outer 50 μm border. The area lying between the solid and dashed line is the 50 μm extra-tumoral region. Numerous blood vessels are seen within the extra-tumoral region (arrowheads) as compared with the intra-tumoral region (arrow). (A, B) Bars = 10 μm

Table I. Sources of data on vessel size and proposed size classification scheme

Vessel type	Vessel diameter (μm)	Vessel size (μm^2)
Published vessel sizes in rat tissues		
Capillary	5.6 \pm 1.9 ^a 5.79 \pm 0.24 ^b (5.3–6.3)	24.62 26.32 (22.2–30.8)
Arteriole	10.69 \pm 1.07 ^b	89.71
Venule	14.41 \pm 2.79 ^b	163.00
Published vessel sizes in rat mammary carcinomas ^c		
Capillaries	<15	<177
Lateral connectors	<5	<19.6
Sinusoidal vessels	15–40	177–1257
Arterioles	40–50	1257–1963
Intra-nodular vessels	20–80	314–5027
Proposed size classification scheme ^d		
\leq Vessel diameter (μm)	Vessel size (μm^2)	% All vessels
\leq 3.57	\leq 10	7.2
>3.57 and \leq 5.64	>10 and \leq 25	25.1
>5.64 and \leq 7.98	>25 and \leq 50	21.5
>7.98 and \leq 9.77	>50 and \leq 75	11.2
>9.77	>75	35.0

^aVessel measurement based on corrosion casts and EM from a 3–5-month-old adult rat heart (mean \pm SD, ref. 20).

^bVessel measurements from resin casts of rat heart (means \pm SEM, ref. 21).

^cVessel measurements from resin casts of DMBA-induced rat mammary carcinomas (22).

^dMeasurements were performed by image analysis on fixed red blood cells in rat mammary carcinomas. The average diameter of a red blood cell was 5.5 μm with an average area of 23.75 μm^2 .

vascular density among carcinomas as reflected in the standard deviations about the means was high when expressed as either vessel count or vessel area per unit area. In general, median values in each vessel size category were lower than the mean; this indicates an asymmetrical distribution of the data. To

Table II. Vascular density of mammary carcinomas as categorized by blood vessel size

Region	Size classification scheme ^a					Total
	≤ 10 μm ²	> 10 and ≤ 25 μm ²	> 25 and ≤ 50 μm ²	> 50 and ≤ 75 μm ²	> 75 μm ²	
Intra-tumoral	Count ^{b,d,e} 8 ^h ± 14 (5)	Count ^{b,d,e} 24 ^g ± 26 (14)	Count ^{b,d,e} 12 ^h ± 8 (11)	Count ^{b,d,e} 5 ^{g,h} ± 5 (5)	Count ^{b,d,e} 11 ^g ± 10 (9)	Count ^{b,d,e} 60 ± 47 (54)
Extra-tumoral ^f	Area ^{c,d,e} 59 ^h ± 103 (31)	Area ^{c,d,e} 396 ^g ± 409 (230)	Area ^{c,d,e} 421 ^h ± 27 (390)	Area ^{c,d,e} 328 ^{g,h} ± 297 (275)	Area ^{c,d,e} 3146 ^g ± 4516 (1744)	Area ^{c,d,e} 4350 ± 4764 (3030)
	Count ^{b,d,e} 41 ^h ± 49 (23)	Count ^{b,d,e} 118 ^{g,h} ± 114 ^g (91)	Count ^{b,d,e} 75 ^h ± 33 (69)	Count ^{b,d,e} 37 ^{g,h} ± 21 (34)	Count ^{b,d,e} 120 ^g ± 47 (112)	Count ^{b,d,e} 391 ± 195 (364)
	Area ^{c,d,e} 297 ^h ± 355 (147)	Area ^{c,d,e} 1966 ^{g,h} ± 1828 (1597)	Area ^{c,d,e} 2657 ^g ± 1098 (2574)	Area ^{c,d,e} 2308 ^h ± 1289 (2132)	Area ^{c,d,e} 61168 ^g ± 47596 (50129)	Area ^{c,d,e} 68397 ± 47568 (55420)

^aAll values are expressed as a mean ± SD, *n* = 36. Median values are in parentheses.

^bCount data are number of blood vessels per unit of assessed area (mm²) falling within the indicated vessel size criteria.

^cArea data are blood vessel area (μm²) per unit of assessed area (mm²) falling within the indicated vessel size criteria.

^dSkewness and kurtosis scores were calculated for all vascular density data for each vessel size category (15). These analyses indicated that the distribution of the data was asymmetrical. The log transformation of these data was approximately normally distributed.

^eComparisons of the adjacent size classes were performed in the MANOVA model on log-transformed data to determine if there were significant differences in vascular density among vessel size classification categories (16).

^fMANOVA was performed on log-transformed data to determine if there were significant differences in vascular density between the intra- and extra-tumoral regions within each vessel size category. All comparisons were statistically different, *P* < 0.03.

^gClasses were statistically different, *P* < 0.05, from the value to the left.

^hClasses were statistically different, *P* < 0.05, from the value to the right.

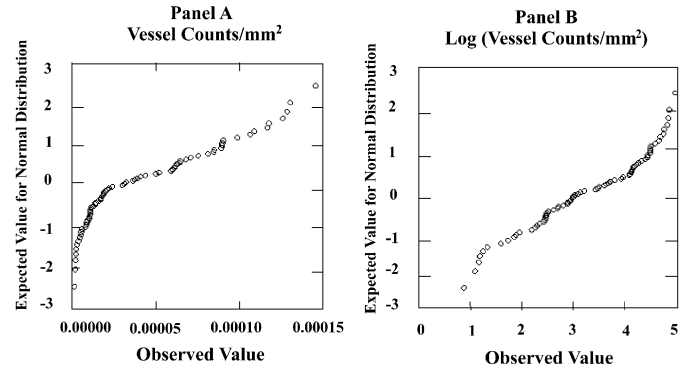


Fig. 2. (A) Normal probability plot of the raw vessel count data for vessel size category 3; this plot is representative of the probability plots for the other vessel size categories. The probability plot is used to determine if a set of data has a normal bell shaped distribution, which is indicated by a linear relationship between expected and observed values. The plot indicates that the vessel count data are not normally distributed. The pattern of this relationship suggested that log transformation would normalize the distribution of these data. (B) A normal probability plot of the log-transformed data for vessel size category 3. The log-transformed vessel count data are approximately normally distributed.

evaluate the degree of skewness and a possible transformation to allow the application of statistical techniques that assume a normal distribution, normal probability plots of the raw (Figure 2A) and log-transformed data (Figure 2B) were produced (15). As indicated by the linear relationship between expected versus observed values (Figure 2B), the log-transformed data were approximately normally distributed and were used for all statistical analyses. There were five size categories of vessel count (or area) data for each tumor; these were analyzed in a repeated measures model, multivariate analysis of variance (MANOVA) (16). Differences among vessel size categories were specified as adjacent differences, that is, we compared size category 1 with size category 2, 2 with 3, and so on. In general, no consistent pattern was observed among vessel size categories in either the intra-tumoral or extra-tumoral region. However, extra-tumoral vascular density (area or count) was significantly higher than intra-tumoral vascular density in all vessel size categories. Collectively, the data in Table II show: (i) that tumor angiogenesis is a highly variable process; (ii) that the density of blood vessels is greater in the area immediately adjacent to a tumor than within the tumor; and (iii) that the frequency of occurrence of blood vessels of different sizes is skewed and the variance increases with the mean. The last observation suggests that the data should be mathematically transformed prior to statistical analyses.

Table III shows the vascular density data, expressed as counts and area, for carcinomas by histological type, and Figure 3 provides a graphic illustration of the same data following its logarithmic transformation. For the data, the following questions were addressed: (i) is there a difference in vascular density among histological types of carcinomas; (ii) holding histologic type constant, is there a difference in vascular density between the intra-tumoral or the extra-tumoral region; and (iii) are the relative differences in vascular density between the intra-tumoral and the extra-tumoral region influenced by tumor histology? Using MANOVA, the between vessel size category effect for histological type was not significant, the between vessel size category effect for region was significant (*P* < 0.0001), and the interaction, i.e. the differences in the ratio of vessel density in the extra-tumoral

Table III. Vascular density by histological type of carcinoma class and location assessed

		Size classification scheme ^a										Total
		$\leq 10 \mu\text{m}^2$		>10 and $\leq 25 \mu\text{m}^2$		>25 and $\leq 50 \mu\text{m}^2$		>50 and $\leq 75 \mu\text{m}^2$		$>75 \mu\text{m}^2$		
Area ^{b,d}	Histological type	Count ^{c,d}	Area	Count	Area	Count	Area	Count	Area	Count	Area	Count
Intra-tumoral	Comedo $n = 4$	1 ± 1	109 ± 70	6 ± 4	456 ± 525	12 ± 14	365 ± 513	6 ± 9	4787 ± 6921	17 ± 23	5725 ± 7974	42 ± 46
	Cribriform $n = 8$	7 ± 6	418 ± 372	26 ± 23	251 ± 155	7 ± 4	125 ± 135	2 ± 2	3261 ± 7891	4 ± 5	4104 ± 8007	46 ± 29
	Mixed $n = 17$	10 ± 19	461 ± 470	28 ± 31	455 ± 250	13 ± 8	405 ± 185	7 ± 3	3174 ± 2531	13 ± 8	4565 ± 2697	71 ± 57
Extra-tumoral	Papillary $n = 7$	10 ± 10	375 ± 394	21 ± 21	511 ± 240	15 ± 7	353 ± 442	5 ± 6	2010 ± 1405	9 ± 6	3324 ± 1927	60 ± 37
	Comedo $n = 4$	18 ± 17	1660 ± 1695	97 ± 104	2597 ± 1173	72 ± 34	2686 ± 898	43 ± 14	91117 ± 88645	134 ± 34	98196 ± 88025	364 ± 138
	Cribriform $n = 8$	84 ± 74	3569 ± 2489	220 ± 157	3758 ± 1054	108 ± 31	2915 ± 1984	47 ± 32	73099 ± 59595	147 ± 55	83944 ± 57440	605 ± 220
Papillary $n = 7$	Mixed $n = 17$	24 ± 31	1342 ± 1143	78 ± 69	2244 ± 891	63 ± 26	1908 ± 941	31 ± 15	45264 ± 23821	98 ± 38	50941 ± 24813	295 ± 133
	Papillary $n = 7$	43 ± 36	1827 ± 1652	109 ± 101	2437 ± 880	70 ± 30	2372 ± 1135	39 ± 19	69041 ± 45303	136 ± 49	75993 ± 44599	397 ± 143

^aAll values are expressed as a mean ± SD.

^bArea data are blood vessel area (μm^2) per unit of assessed area (mm^2) falling within the indicated size criteria.

^cCount data are number of blood vessels per unit of assessed area (mm^2) falling within the indicated vessel size criteria.

^dArea and count data were subjected to multivariate analyses of variance. A highly significant difference ($P < 0.0001$) was noted between intra-tumoral versus extra-tumoral vascular density irrespective of the manner in which it was measured (area or count). No statistically significant differences were associated with histological classification.

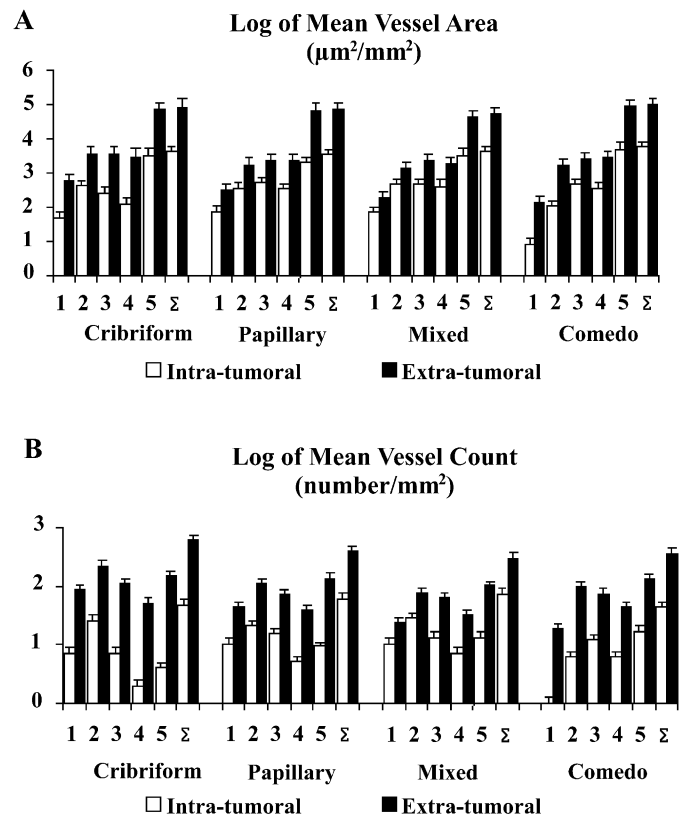


Fig. 3. (A) Vessel area by vessel size category and histological type of carcinoma. Vessel size categories on the x-axis are: 1, $\leq 10 \mu\text{m}^2$; 2, >10 and $\leq 25 \mu\text{m}^2$; 3, >25 and $\leq 50 \mu\text{m}^2$; 4, >50 and $\leq 75 \mu\text{m}^2$; 5, $>75 \mu\text{m}^2$. Values are means ± SEM of log-transformed vessel area data. (B) Vessel counts by vessel size category and histological type of carcinoma. Vessel size categories on the x-axis are: 1, $\leq 10 \mu\text{m}^2$; 2, >10 and $\leq 25 \mu\text{m}^2$; 3, >25 and $\leq 50 \mu\text{m}^2$; 4, >50 and $\leq 75 \mu\text{m}^2$; 5, $>75 \mu\text{m}^2$. Values are means ± SEM of log-transformed vessel count data.

versus the intra-tumoral region across vessel size categories, between the two effects was significant ($P < 0.02$ in both area and count data). This interaction is illustrated in Figure 3 where it is apparent that the differences in the ratio of vessel density in the extra-tumoral versus the intra-tumoral region across vessel size categories tends to be greater in cribriform and comedo carcinomas than in papillary or mixed carcinomas. Thus, the data shown in Table III and Figure 3 indicate that: (i) vascular density in the extra-tumoral region is greater than in the intra-tumoral region in all histological types of mammary carcinoma; and (ii) that the magnitude of these differences may vary among histological types of tumors and within vessel size categories.

Census counting provided an extensive amount of information on the density of blood vessels expressed as number or area of vessels within five different vessel size categories. Of interest is whether the area and count data provide independent information. In order to investigate this question, the data set used to derive the values shown in Table II was subjected to correlation analyses. These analyses were not formulated until the evaluation in Table III was completed. The reason for this is that it was important to determine first how vascular density was affected by either location of the blood vessels assessed (intra- versus extra-tumoral region) or the histological type of carcinoma. As vascular density is not dependent on histological type, all density data were pooled across type; however, the large difference between intra- versus extra-tumoral vascular

Table IV.

Vessel size category	$\leq 10 \mu\text{m}^2$		$>10 \text{ and } \leq 25 \mu\text{m}^2$		$>25 \text{ and } \leq 50 \mu\text{m}^2$		$>50 \text{ and } \leq 75 \mu\text{m}^2$		$>75 \mu\text{m}^2$	
	IT ^a	ET ^b	IT	ET	IT	ET	IT	ET	IT	ET
(A)										
$\leq 10 \mu\text{m}^2$	1.00	1.00	–	–	–	–	–	–	–	–
$>10 \text{ and } \leq 25 \mu\text{m}^2$	0.82*	0.88*	1.00	1.00	–	–	–	–	–	–
$>25 \text{ and } \leq 50 \mu\text{m}^2$	0.23	0.46	0.30	0.61*	1.00	1.00	–	–	–	–
$>50 \text{ and } \leq 75 \mu\text{m}^2$	–0.05	–0.25	0.05	–0.14	0.70*	0.44	1.00	1.00	–	–
$>75 \mu\text{m}^2$	–0.20	–0.04	–0.09	0.01	0.50	0.26	0.60*	0.18	1.00	1.00
(B)										
$\leq 10 \mu\text{m}^2$	1.00	1.00	–	–	–	–	–	–	–	–
$>10 \text{ and } \leq 25 \mu\text{m}^2$	0.87*	0.93*	1.00	1.00	–	–	–	–	–	–
$>25 \text{ and } \leq 50 \mu\text{m}^2$	0.37	0.56*	0.38	0.68*	1.00	1.00	–	–	–	–
$>50 \text{ and } \leq 75 \mu\text{m}^2$	–0.02	–0.20	0.05	–0.16	0.72*	0.38	1.00	1.00	–	–
$>75 \mu\text{m}^2$	–0.15	–0.10	–0.07	–0.06	0.64*	0.46	0.76*	0.59*	1.00	1.00
(C)										
Correlation coefficient	0.97*	0.99*	0.96*	0.96*	0.99*	0.99*	0.95*	0.99*	0.92*	0.66*
(D)										
Vessel size category	$\leq 10 \mu\text{m}^2$		$>10 \text{ and } \leq 25 \mu\text{m}^2$		$>25 \text{ and } \leq 50 \mu\text{m}^2$		$>50 \text{ and } \leq 75 \mu\text{m}^2$		$>75 \mu\text{m}^2$	
	Area ^c	Count ^d	Area	Count	Area	Count	Area	Count	Area	Count
$\leq 10 \mu\text{m}^2$	0.88*	0.88*	0.75*	0.75*	0.37	0.08	–0.35	–0.32	–0.12	–0.36
$>10 \text{ and } \leq 25 \mu\text{m}^2$	0.79*	0.84*	0.79*	0.81*	0.42	0.01	–0.32	–0.31	–0.05	–0.35
$>25 \text{ and } \leq 50 \mu\text{m}^2$	0.09	0.43	–0.04	0.47	0.04	0.03	0.20	–0.29	0.11	–0.31
$>50 \text{ and } \leq 75 \mu\text{m}^2$	–0.28	–0.35	–0.30	–0.32	–0.26	0.19	0.08	0.08	0.12	–0.17
$>75 \mu\text{m}^2$	–0.31	–0.28	–0.30	–0.29	–0.27	–0.02	0.16	–0.15	0.03	–0.04

(A) Correlation coefficients for vessel density ($\mu\text{m}^2/\text{mm}^2$) across vessel size categories.

(B) Correlation coefficients for vessel density (count/ mm^2) across vessel size categories.

(C) Correlation coefficients among vessel size category for vessel area versus vessel count.

(D) Correlation coefficients within vessel size categories for the relationship between intra-tumoral versus extra-tumoral vessel density (area or counts).

Values are Pearson correlation coefficients. Correlation coefficients with asterisks are statistically significant, $P < 0.05$.

^aIntra-tumoral vascular density (IT).

^bExtra-tumoral vascular density (ET).

^cArea data are blood vessel area (μm^2) per unit of assessed area (mm^2) falling within the indicated vessel size category.

^dCount data are number of blood vessels per unit of assessed area (mm^2) falling within the indicated vessel size category.

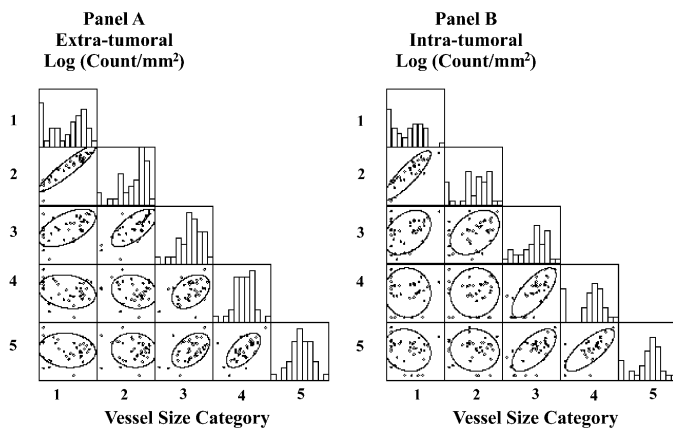


Fig. 4. Graphical presentation of the correlations among vessel counts of different vessel size categories in the extra- (A) and the intra-tumoral (B) region. The more elliptical and narrow plot, the stronger is the correlation. A plot that is a circle would indicate the lack of correlation between two variables. Log-transformed data were used in these analyses.

density provided the rationale for analyzing the vascular density within each region separately. The results of the correlation analyses are shown in Table IV and Figure 4. The first question asked was: within assessment region, i.e. intra- or extra-tumoral region, how is the density of small vessels related to the density of larger vessels? As indicated by the correlation coefficients shown in Table IV(A), the density of vessels in adjacent size classes are correlated, with the strongest correlation among sizes $\leq 25 \mu\text{m}^2$ ($P < 0.01$). However, as

illustrated in Figure 4, the correlation of the density of these smaller vessels with vessels $>25 \mu\text{m}^2$ is low and becomes increasingly negative with increasing vessel size, i.e. the smaller the number of large vessels, the higher the number of small vessels. However, the majority of the correlation coefficients reported were not statistically significant. A similar pattern of correlation was observed for vessel density expressed as vessel counts per unit area [Table IV(B)]. Interestingly, as shown in Figure 4, correlations among vessel size categories were weaker in the extra-tumoral versus the intra-tumoral region.

Given the similarity of the pattern between vessel density area and count correlation coefficients, the second question asked was: within assessment location, is vessel area correlated with vessel count within the same size category? As shown in Table IV(C), a high degree of correlation was observed between vascular density expressed as vessel counts and vessel area within each size category ($P < 0.001$). This finding, which is illustrated in Figure 5 shows the linearity of the relationship between vessel count and vessel area data, indicating that the data provide virtually the same information.

The third question addressed was: is vascular density data in the intra-tumoral region correlated with vascular density in the extra-tumoral region? As shown in Table IV(D), the only positive associations observed between intra- and extra-tumoral vessel density were for blood vessels $\leq 25 \mu\text{m}^2$. None of the other correlation coefficients reported were statistically significant. The data indicate that the density of intra-tumoral vessels $\leq 25 \mu\text{m}^2$ is highly correlated with the density of extra-

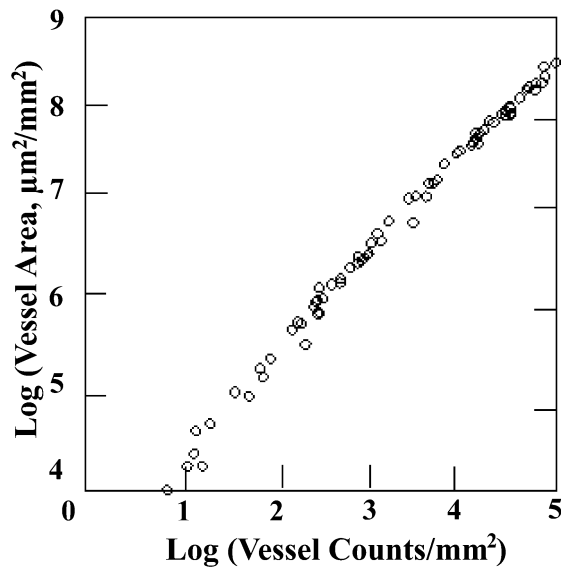


Fig. 5. Plot of the relationship between vessel count and vessel area data in vessel size category 3. The linearity of this relationship indicates that similar information is provided by both variables. Linear plots were also obtained when this analysis was performed on data for all other vessel size categories.

tumoral vessels of the same size. However, the density of smaller vessels in one region does not reflect the density of larger vessels ($>25 \mu\text{m}^2$) in the opposite region, nor does the density of larger vessels in one region reflect the density of smaller vessels in the adjacent region.

In summary, the data presented in Table IV and Figures 4 and 5 indicate: (i) that distinct information is obtained by measuring blood vessels in all size categories; (ii) that similar information is obtained by measuring vessel counts and vessel area; and (iii) that in general, extra-tumoral vessel density is unrelated to the density of intra-tumoral vessels $>25 \mu\text{m}^2$.

Discussion

The MNU-induced mammary carcinogenesis model in the rat is widely used to evaluate the cancer preventive activity of chemopreventive agents, various nutrients and other dietary factors. This model also is used to study how these agents exert their effects. While considerable attention has been directed to investigating mechanisms directly linked to cell proliferation and apoptosis, there is now increasing recognition that some cancer preventive agents may act by blocking the process of angiogenesis. Based on the early work of Gullino *et al.* (3–5) in which mammary tissue from MNU-treated rats as well as MNU-induced mammary tumors were observed to stimulate vascularization in the rabbit iris angiogenesis assay, it appears that the MNU model is well suited for the investigation of agents that may modulate the process of neovascularization. However, there is a paucity of information about the vascular profile of autochthonously developing mammary tumors in any experimental model system. In the following paragraphs a number of factors are discussed that should be considered in collecting and reporting data from experiments in which a primary endpoint is the measurement of blood vessel density as an indicator of angiogenic activity.

Rationale for vessel size categories

While a number of *in vitro* and *in vivo* assays are available to assess angiogenic activity *per se*, efforts to assess angiogenesis

in situ necessarily measure the outcome of the process, i.e. the vascular density of a tissue or tumor, with the assumption that greater vascular density equates to greater angiogenic activity (17). Because no quantitative data are available on the vascular profile of rat mammary tissue or tumors, we chose to use a census counting approach to measure the size and number of all blood vessels identified by CD31 immunostaining. The rationale for using this epitope to detect endothelial cells is that CD31 allows greater discrimination, than does staining for the more commonly used Factor VIII epitope, of blood vessels with areas $<25 \mu\text{m}^2$, and demonstrates crisp staining of blood vessels in mammary tissue and tumors with minimal background and excellent preservation of tissue architecture (13). The blood vessel size and count data were used to compute the vascular density of 36 mammary carcinomas induced by MNU. The method of data collection used, which is described in detail in ref. 13, provides the opportunity to divide blood vessels into as many categories as desired based on vessel size. Such divisions, based on measurement data, to our knowledge, have not been reported previously. It was decided to divide vessels into five size categories. The smallest size category, $<10 \mu\text{m}^2$, is mostly likely to represent newly forming blood vessels, as the diameter of these vessels is $\sim 50\%$ or less than that of a rat's red blood cell; 7.2% of all vessels measured fell into this category (Table I). The next three categories reflected vessels of increasing size, the density of which would be likely to reflect recent angiogenic activity. These categories, >10 and ≤ 25 , >25 and ≤ 50 , >50 and ≤ 75 accounted for 25.1, 21.5 and 11.2% of all blood vessels. The final category, vessels $>75 \mu\text{m}^2$, contained 35.0% of all vessels counted. While it was possible to further subdivide this category, we judged that vessels with these dimensions were likely to reflect the established vascular supply, and consequently there was no clear rationale for dividing these vessels into additional size categories. Whether there are specific size categories that would be more informative than others in studying the effects of various cancer preventive agents on angiogenesis remains to be determined, but the evidence presented here gives little indication that differential effects will be observed.

Intra-tumoral vascular density

The data shown in Table II indicate that there is considerable variability among mammary carcinomas in intra-tumoral vascular density reported in terms of either counts or area. This is apparent from the fact that the standard deviations about the mean frequently equalled or exceeded the mean values reported. This variability, which was observed in all vessel size categories, was not anticipated as the 36 mammary carcinomas had similar biological ages; such variability is likely to reflect, at least in part, a complex interplay among the expression of genes controlling vascularization and the local intra- and extra-tumoral environments in which angiogenic activity is manifest. Because of the variability in vascular density, the agent under study must have a large effect on neovascularization for statistical significance to be determined, and/or a relatively large number of carcinomas will have to be studied to detect biological effects that are statistically significant. Table II provides means and standard deviations about the mean; this information, along with the covariance matrix for the size classes, can be used for statistical power calculations to determine appropriate sample size for studies in which blood vessel density is used as an endpoint for angiogenesis. An

additional consideration revealed by the data in Table II is that the distributions of vessel densities, by area or count, in general were asymmetrical within each vessel size category. Both probability plot analyses and measurement of the skewness and kurtosis of the data distributions confirmed asymmetry. While this result was suggested in a recent publication (13), it was hoped that division of vessels into relatively small size intervals would decrease the skewness of the distributions; it did not. However, the log transformation of the data was approximately normally distributed (Figure 2B). This permitted data analyses using robust parametrical statistical procedures. Both factors, the asymmetry of the raw data and the availability of a simple mathematical transformation, are important considerations in the development of a data analysis plan.

The data in Table II left a key question unanswered, namely was the variability observed in vascular density (Table II) associated with the histological type of carcinoma? As reported in ref. 18, there are four primary types of mammary carcinomas induced by MNU. They are: cribriform, papillary, mixed cribriform-papillary and comedo. Inspection of the data shown in Table III indicates that the vascular density data remain highly variable when categorized by histological type of carcinoma. Using multivariate analysis of variance on the log-transformed data to evaluate the data shown in Table III, it was determined that while numerical differences did exist in vascular density among tumor types, some of these differences were of sufficient magnitude to be statistically significant within vessel size category (measured statistically as an interaction), but not between vessel size categories. This observation suggests that examining vascular density by histological type could be helpful in explaining differential effects of cancer preventive agents on angiogenesis in the extra- versus the intra-tumoral region even though there is no independent effect on tumor vascularization that is attributable to tumor histology.

Following the multivariate analyses, all carcinoma data were subjected to the correlation analyses reported in Table IV. The rationale underlying these analyses was to determine if all data obtained from the census counting procedure, which is both labor intensive and time consuming, provide independent information. Independence was assessed via correlation analysis based on the premise that two variables that are highly linearly related provide similar information about a treatment effect. In Table IV(A) and (B), the results show lack of statistically significant correlations among most of the five vessel size categories; however, as might be anticipated, correlations were highest and at times significant for adjacent size categories. This is an anticipated result given that division of vessels into size categories was arbitrary as discussed in a preceding paragraph. In studying the patterns of correlation, a trend for the density of larger vessels to be inversely associated with the density of smaller vessels was noted and is consistent with the hypothesis that limitations in the supply of oxygen and nutrients to developing clones of cells are factors that induce angiogenesis (1). However, as correlation coefficients more than one size category apart were not statistically significant, it can be concluded that obtaining data on vessels of all sizes is providing useful information. On the other hand the correlation analysis reported in Table IV(C) was performed after noting the similarity of results in Table IV(A) and (B), for vessel density expressed as area or counts. In the correlation analyses resulting in the coefficients shown in Table IV(C), the question asked was, does reporting vessel density in these

two ways (vessel area versus vessel count) provide different information? As illustrated in Figure 5, the fact that the correlation coefficients were extraordinarily high indicated little additional quantitative information is provided by reporting vessel density as both counts per unit area and vessel area per unit area. Thus, while this does not affect the data collection process, in general there appears to be little reason for reporting data in both formats unless the investigator judges that some qualitative aspect of the data set merits the presentation of both parameters. Nonetheless, it was observed that for the largest vessel size class in the extra-tumoral region that the correlation between area and count was only 0.66; consequently, if anti-angiogenesis was manifest initially in the density of large vessels, the area measurement could be more sensitive to change than counts. Thus, statistical analyses of both types of data are recommended with the decision on the format of the data to be presented based on the results of those analyses.

Extra-tumoral vascular density

While some reports hypothesize that tumors vascularize by growing around established blood vessels, in essence co-opting a vascular supply, other evidence indicates that tumor epithelial cells and/or adjacent stromal cells secrete factors that elicit blood vessel growth and the ultimate penetration of these vessels into the developing mass of tumor cells (19). Thus, it might be anticipated that useful information about the process of angiogenesis and its modulation could be derived not only from evaluating intra-tumoral vascularization, but also the vascularization of the tissue immediately adjacent to a developing tumor. Interestingly, no data could be identified in the literature in which the extra-tumoral as well as the intra-tumoral blood supply were assessed. As shown in Table II, extra-tumoral vascular density exhibits the same distributional characteristics as the intra-tumoral data. Thus, the same discussion points included under intra-tumoral vascular density apply to the measurement of the extra-tumoral blood supply. However, what was notably different was the magnitude of the extra-tumoral vascular density; it was approximately twice that of the intra-tumoral blood supply and, as shown in Table III, this difference was observed across carcinomas of all histological types and was consistently observed in all vessel size categories. These observations raised the interesting question of whether the information provided by assessment of the extra-tumoral compartment was related to intra-tumoral vascular density. The data shown in Table IV(D), provide clear evidence that the data from one compartment is not highly correlated with the other, indicating that independent information is provided by the assessment of both compartments. Therefore, it would appear that by studying both aspects of vascular supply that the most complete picture of tumor vascularization, and by implication, angiogenesis and its modulation could be obtained.

Summary

It appears that the process of angiogenesis as reflected in the pattern of vascularization of chemically induced mammary carcinomas is highly variable. Given that patterns of intra-versus extra-tumoral vascular density are not, in general, highly correlated, it is likely that different factors, environmental and/or genetic, are influencing these processes, and that by studying them both, the clearest picture of tumor-induced angiogenesis

can be obtained. Collectively the data provide a complete description of vascularization in autochthonously growing mammary tumors, and a reference for comparison in studies of various cancer preventive agents that modulate the angiogenic process.

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