Mast Cells and Tumors

The Specific Enhancement of Tumor Proliferation in Vitro

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Mast cells were found to be unique among the peritoneal leukocytes by virtue of their capacity to enhance profoundly the proliferation of a variety of tumors *in vitro*. This phenomenon occurs at mast cell/tumor ratios which reflect the stoichiometry of host cell/tumor relationships *in vivo*. The growth factor was found to reside in mast cell granules and was identified as heparin by sequential purification and enzymatic degradation. This cellular interaction was tumor-specific, although isolated granules could enhance fibroblast proliferation. The findings are discussed in relation to previous morphologic studies, reports of *in vitro* mast-cell–mediated tumor cytotoxicity, and the role of mast cells in angiogenesis and connective tissue proliferation. (Am J Pathol 1985, 119:57–64)

THE OCCURRENCE of inflammatory reactions at the periphery of growing tumors has long been taken as evidence of a host defense mechanism.[1] However, the demonstration of immunostimulation of tumor growth,[2] inhibition of growth of transplanted tumors by antiinflammatory drugs,[3,4] and the enhanced growth of tumors transplanted into sites of inflammation[5] has led to the formulation of another paradigm of the host/tumor interaction. For further testing and definition of this alternative hypothesis of leukocyte-mediated enhancement of tumor growth, a variety of cells from the peritoneal cavity were examined for their capacity to stimulate the cellular proliferation of tumors *in vitro*.

Materials and Methods

Animals

CBA/H mice were obtained from the Gore Hill Research Laboratories (NSW, Australia), and congenic CBA/H-T6 mice and DA rats were obtained from the Blackburn Animal House (University of Sydney). The animals used in experiments were of either sex and between 8 and 12 weeks of age.

Tumors

The sarcomas D7, D8, and D9 were induced in DA rats by subcutaneous injection of 2 mg of 20-methylcholanthrene in olive oil. HK 8 was similarly induced in CBA/H mice with 1 mg of 20-methylcholanthrene. ST-1 and ST-2, both adenocarcinomas, and SP-1, a squamous carcinoma, arose spontaneously in DA rats. All the tumors had been passaged *in vivo* and were stored in liquid nitrogen. All cultures were passaged at least twice *in vitro* prior to use in experiments.

Nonneoplastic Cells

Fibroblasts were cultured for explants of subcutaneous fascia from adult DA rats. 3T3 BALB/c cells were obtained from Commonwealth Serum Laboratories (CSL, Parkville, Australia).

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All the cultures were maintained in 75-sq cm flasks (Lux, catalog no. 5375, Miles Laboratories, Naperville, Ill) in 20 ml of Dulbecco's Modified Eagle's Medium (DME, GIBCO Laboratories, Grand Island, NY) with 10% fetal calf serum (FCS, Flow Laboratories, Sidney, Australia), 100 U/ml penicillin, and 100 μg/ml streptomycin (Glaxo, Boronia, Australia). The cultures were passaged with the use of 0.05% trypsin (Trypsin 1:250, Difco Laboratories, Detroit, Mich) with 0.5 mM EDTA, and maintained *in vitro* for up to 12 weeks.

Harvesting and Separation of Peritoneal Cells

Mice were sacrificed by cervical dislocation; rats were exsanguinated by severing of the cervical vessels, under deep ether anesthesia. Aseptic peritoneal lavage was performed with the use of a Tris, albumin, EDTA buffer, pH 7.6,[6] with the substitution of bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, Mo) for the human albumin in the original formulation. This buffer was also used for all metrizamide gradient separations.

Rat neutrophil-, eosinophil-, and mast-cell-enriched fractions were prepared by centrifugation through a discontinuous gradient (18%, 20%, 22%, 23%, 24%, and 26%) of metrizamide (grade I, Sigma).[7] A macrophage-enriched population was obtained by the use of a discontinuous gradient (47.5%, 50.8%, and 52.5%) of isotonic Percoll (Pharmacia, Uppsala, Sweden) in DME with 10% FCS (see Timonen et al[8] and E. Farram, unpublished observations). In subsequent experiments, mast cells from rats and mice, as appropriate to the tumor used, were isolated with the use of a single 22.6% metrizamide step.[6]

Enriched populations were washed three times in DME with 1% FCS. The cells were cytocentrifuged (Cytospin, Shandon Southern Ltd., Camberley, England) and stained with Dif Quik (AHS, Sidney, Australia), and a differential count was performed on 200 cells. Viability was assessed by the exclusion of 0.1% nigrosin. The purity and viability of mast cells, in later experiments, was assessed by phase-contrast microscopy using a ×40, medium-absorption, negative-phase objective (Olympus Optical Co., Tokyo, Japan).

Preparation of Mast-Cell Granules

Rat mast cells, purified as above, were centrifuged and resuspended in 0.34 M sucrose. The cells were sonicated in intermittent bursts in a sonication bath (B12, Branson, Conn) at 4 C. The disruption was monitored by phase-contrast microscopy. The sonicate was diluted in 0.34 M sucrose, centrifuged at $200g_{max}$ for 7 minutes, and the supernatant was recentrifuged at $1500g_{max}$ for 45 minutes at 4 C.[9] The pellet was resuspended in DME, and 400 granules were counted in a hemocytometer by phase-contrast microscopy (×720 magnification). The granules were left untreated, or the perigranular membrane was removed by lysis with distilled water.[10] Osmolarity was restored by the addition of four times DME or dialysis against three changes of 500 volumes of DME, for 18 hours each, at 4 C.

Separation of Mast-Cell Proteoglycan and Granule Protein

Mast-cell granules, suspended in 1 M NaCl, 0.01 M Tris, pH 9.0, were frozen and thawed three times and applied to a 10 × 1-cm column of Dowex AG1-X2, chloride form, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif) equilibrated in the same buffer. The column was washed with 30 ml of buffer followed by 30 ml of 3 M NaCl.[11] Both these fractions were concentrated by vacuum dialysis at 4 C against five changes of 0.15 M NaCl. The proteoglycan content was measured by a variation of the Azure A (Sigma) metachromasia assay,[12] the decrease in absorbance at 620 nm being measured with a spectrophotometer, with the use of commercial heparin (150–160 U/mg, CSL) as a standard. Protein was measured with the Folin phenol reagent,[13] with BSA as a standard.

Further purification of the proteoglycan fraction was performed by incubation with 10 mg/ml of insoluble trypsin (Trypsin −30 Enzygel, Boehringer Mannheim, Sidney, Australia) in the presence of 0.015 M $CaCl_2$ in a shaking water bath at 37 C for 30 minutes, followed by centrifugation ($1000g_{max}$, 7 minutes) and dialysis against 0.15 M NaCl. Heparin was further purified by treatment of 50 μg trypsin-treated material with 5 units of chondroitinase ABC (Sigma) at 37 C, pH 8.0, for 30 minutes, in the presence of BSA,[14] chromatographed on a Dowex AG1-X2 column for removal of the proteins, and concentrated by vacuum dialysis.

Heparinase Treatment

Mast-cell heparin, purified as above, was diluted in DME and incubated at a final concentration of 20 μg/ml with 2 U/ml heparinase (Sigma) at 30 C, pH 7.0, for 3 hours.[15] Controls (DME with and without heparin) were incubated with either buffer or heparinase under the same conditions.

Tumor Cell Proliferation Assays

Cells were harvested from confluent cultures with the use of trypsin–EDTA, FCS was added to 10% to neutralize the trypsin, and the cells were washed three times

with DME + 1% FCS. The same batch of FCS (29101814, Flow) was used for all experiments. The cells were counted, viability was assessed with nigrosin (generally >98% viable cells), and 10^4 viable tumor cells, with or without leukocytes or leukocytes alone, were plated in microculture plates (96 flat-bottom wells, Linbro catalog no. 76.003.05, Flow) in 100-μl aliquots of DME with 1% FCS. The cells were cultured for 48 hours at 37 C in 5% CO_2, 10 μl of DME containing 0.5 μCi of ^{3}H-thymidine (6.7 Ci/mMol, New England Nuclear, Boston, Mass) being added to each well for the last 2 hours of culture. The medium was then aspirated, and each well was washed with phosphate-buffered saline (PBS) prior to incubation in 0.3% trypsin in EDTA for 30 minutes. In the case of D8, a loosely adherent tumor, the PBS wash was omitted. The cultures were then harvested onto glass fiber disks with an automatic harvester (Skatron AS, Heggtoppen, Norway).

Thymidine incorporation was measured by liquid scintillation counting, the results being expressed as counts per minute (cpm) per well. All cultures were performed in quadruplicate, and all experiments, at least in duplicate.

Mast-cell granules were assayed similarly. Soluble components, including commercial heparins, (porcine mucosal, preservative-free, 1000 U/ml, CSL; 5000 U/ml, David Bull Laboratories, Mulgrave, Australia; 5000 U/ml, Weddel Pharmaceuticals, Clwyd, UK) and chondroitin sulfate (from whale cartilage, Sigma), were membrane-filtered (0.2 μ, Acrodisc, Gelman Sciences, Sidney, Australia) prior to analysis and assay.

For the direct measurement of proliferation, cells were plated in tissue-culture slide chambers (Lab-Tek, catalog no. 4808, Miles Laboratories) at 10^4 cells/sq cm, in DME 1% FCS with and without mast-cell heparin. After 3 days' incubation half the medium was removed and replaced with fresh medium with the appropriate heparin concentration.

At 2, 4, and 6 days the medium was discarded; and the cells were air-dried, fixed in methanol, and stained with 0.05% aqueous toluidine blue, pH 4.2. Cell counts were performed in quadruplicate with the use of ×10 graticule eyepiece. Approximately 400 cells were counted in each case, and the results are expressed as cells per square centimeter.

Heparin Release Assay

Mast cell proteoglycans were labeled by the administration of three doses of 250 μCi of Na_2 $^{35}SO_4$ (Amersham, Sidney, Australia) intraperitoneally to rats at 2-day intervals.[16] Two to 3 days after the final dose, the peritoneal mast cells were purified as above, and 10^4 mast cells were cultured in 1 ml of 1% FCS DME, alone or with 10^5 D8 tumor cells or fibroblasts for 24 hours. The supernatant was recovered and centrifuged at $400g_{max}$ for 10 minutes. The proteoglycans in the cell-free supernatant were precipitated with cetylpyridinium chloride[17] and measured on a LKB Rackbeta II liquid scintillation counter, (Linbrook International, Sidney, Australia) using external standard quench correction. Results were expressed as disintegrations per minute of cetylpyridinium chloride precipitable ^{35}sulfur released per culture.

Statistical Methods

Stimulation indices were calculated as:

$$\frac{\text{(Tumor + leukocytes) cpm} - \text{leukocytes cpm}}{\text{Tumor cpm} - \text{Background cpm}}$$

The mean for each variable was used. For granules and soluble factors "background cpm" was substituted for the second expression in the numerator.

Statistical significance was variously assessed by the two-tailed Student t test[18] and one- and two-way analysis of variance (ANOVA),[19] with the use of a small computer (PB-100, Casio, Tokyo, Japan).

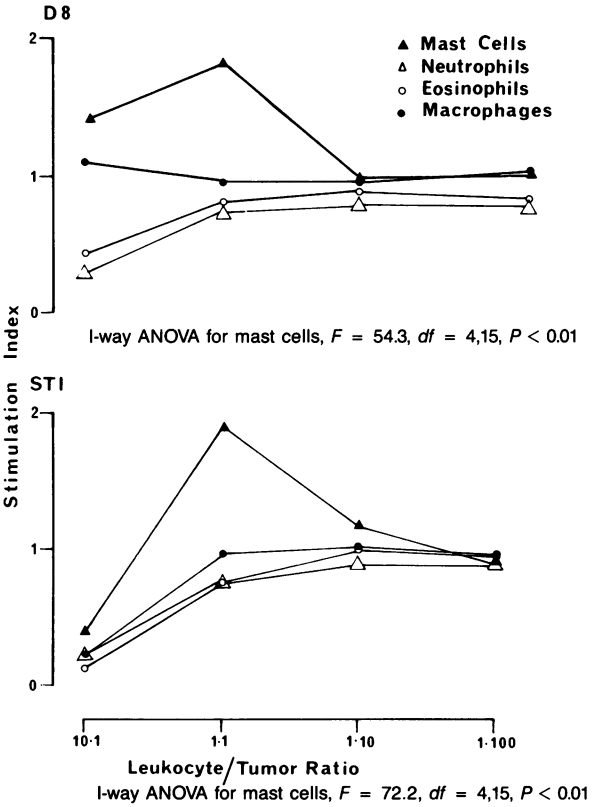

Figure 1—Proliferation of tumor cells in the presence of graded numbers of purified peritoneal leukocytes.

Table 1—The Effect of Mast-Cell Coculture on a Range of Rat Tumors and Nonneoplastic Cells

	Thymidine uptake (cpm, mean ± SD) Mast cell/tumor ratio			
Cell line	0	1:1	1:10	1:100
Rat				
SP-1	130 ± 36	969 ± 59*	2169 ± 257*	2974 ± 96*
ST-1	1067 ± 40	1988 ± 121*	1259 ± 79	978 ± 22
ST-2	17082 ± 531	22767 ± 715*	24800 ± 582*	23093 ± 752*
D7	10063 ± 790	8933 ± 313	9293 ± 883	9155 ± 958
D8	1265 ± 59	2516 ± 183*	1566 ± 124†	1128 ± 51
D9	56 ± 7	234 ± 26*	124 ± 19‡	498 ± 219
Fibroblast	565 ± 61	106 ± 24*	219 ± 33‡	345 ± 47†
Mouse				
HK8	2493 ± 893		4061 ± 382†	3171 ± 529
3T3	160 ± 18		143 ± 10	116 ± 31†

* $P < 0.001$.
† $P < 0.05$.
‡ $P < 0.01$.

Table 2—The Influence of Mast-Cell Granules on Tumor and Fibroblast Proliferation

	Thymidine uptake (mean cpm)						
	Number of granules/culture						
	1×10^4	4×10^4	1×10^5	4×10^5	1×10^6	4×10^6	Control
D8							1683
Untreated	1739	1784	1799	2341	3843	4128	
H_2O-treated	1684	1719	1701	1904	2515	3855	
+ Dialysis	1758	1592	1667	1898	2473	3190	
			*F columns$_{[4,8]}$ = 20.453, $P < 0.01$ F rows$_{[2,8]}$ = 3.882, NS				
DA fibroblasts							452
Untreated	445	487	588	728	839	1296	
H_2O-treated	416	510	603	709	825	1062	
+ Dialysis	466	530	545	746	886	1216	
			*F columns$_{[4,8]}$ = 75.538, $P < 0.01$ F rows$_{[2,8]}$ = 1.229, NS				

* ANOVA was performed with 0, 1×10^5, 4×10^5, 1×10^6, 4×10^6 granules.

Results

Neutrophils were recovered at the 18/20% metrizamide interface (80% neutrophils, 2% lymphocytes, 18% eosinophils) and eosinophils at the 20/22% interface (75% eosinophils, 20% neutrophils, 5% lymphocytes). The pellet consisted of 98% mast cells (1% lymphocytes, 1% eosinophils). Macrophages were obtained at the 47.5/50.8% Percoll interface (80% macrophages, 7% lymphocytes, 6% eosinophils, 5% neutrophils, 2% mast cells). Mast cells were purified to 99% from the same pooled peritoneal washout, with the use of a single metrizamide step, with a yield of 40–50%. Cell viability exceeded 96% in every case.

In co-culture with tumor cells, at leukocyte/tumor ratios of 10:1 to 1:100, the capacity to enhance tumor proliferation was found solely in the mast-cell-enriched population (Figure 1). The thymidine uptake of leukocytes alone was of the same order as "background cpm," reflecting either the terminal differentiation or the nonadherent nature of these cells.

Table 1 shows the range of tumors stimulated by mast cells. All the spontaneous carcinomas and three out of four of the methycholanthrene-induced sarcomas showed significantly enhanced proliferation. This phenomenon occurred with both rats and mice, while neither adult fibroblasts nor 3T3 cells were stimulated; nor did any of the other populations, as in Figure 1, stimulate fibroblast proliferation (data not shown).

This proliferative effect could also be produced by the addition of isolated mast-cell granules to the cultures, as demonstrated in Table 2. Stimulation was demonstrable irrespective of lysis of the perigranular membrane or extensive dialysis of the granules, indicating the presence of the growth factor in mast-cell

Table 3—Lack of Effect of Heat-Inactivation of FCS on Mast-Cell-Induced Tumor Proliferation

Thymidine Uptake (cpm, mean ± SD)			
HK8 (10^4 cells)	+ Mast cells (10^2)	P*	Stimulation index
2564 ± 208	3201 ± 274	=0.005	1.25 Untreated serum
609 ± 68	1165 ± 180	<0.001	2.0 Heat-inactivated serum

* Student t test.

granules, akin to the form of granule found after mast-cell degranulation,[20] and its macromolecular nature. Contrary to the findings with intact mast cells, fibroblasts also responded to the addition of mast-cell granules by enhanced proliferation.

The mechanism of release of this factor in mast cell/tumor cocultures was not complement-mediated mast-cell degranulation, because, as shown in Table 3, heat-inactivation of the FCS (56 C, 30 minutes) did not reduce the proliferative effect, although the intrinsic stimulatory activity of the FCS was reduced.

Ion exchange chromatography yielded two fractions: the 1 M NaCl eluate consisting mainly of protein with less than 4% proteoglycan and the 3 M eluate, comprising 87% proteoglycan and 13% protein. Figure 2 shows that tumor proliferation was enhanced by the 3 M eluate at 100 ng/ml, no activity being detected in the 1 M fraction. The polysaccharide nature of the growth factor was also indicated by its resistance to trypsin, as shown in Figure 3. Examination of commercially available glycosaminoglycans revealed that some commercial heparin had some growth-promoting activity, but only detectable at an order of magnitude higher concentration than mast-cell proteoglycan, even then the effect being less than that of mast-cell heparin (Table 4). Chondroitin sulfate exhibited no activity, nor did chondroitinase treatment of the mast-cell proteoglycan preparation reduce its growth-stimulatory activity. Incubation of the trypsin-treated proteoglycan with chondroitinase ABC actually increased the specific activity of material, probably because of a small shift in the dose-response curve due to removal of inactive, but metachromatic, chondroitin sulfate (Figure 4).

That the growth factor was indeed heparin was confirmed by the abolition of all the growth-promoting activity by the specific enzymatic degradation of heparin (Table 5). Heparinase alone had no inhibitory effect, but rather induced a small but statistically significant enhancement of proliferation. The proliferative effect of mast-cell heparin, as measured by thymidine uptake, was confirmed by direct cell counting (Figure 5), there

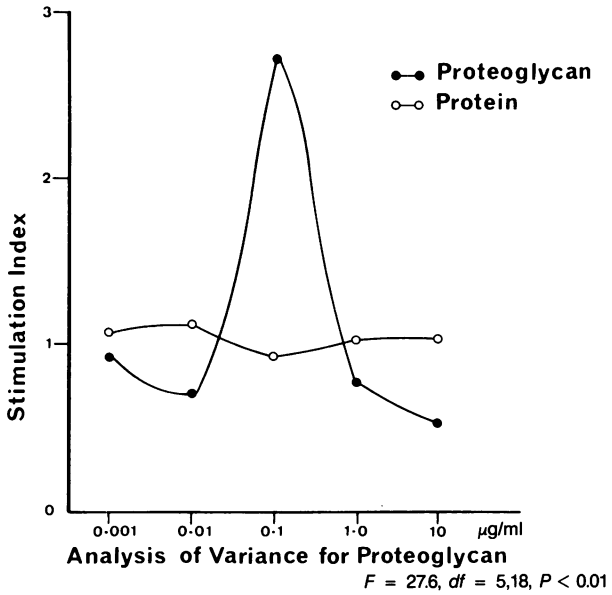

Figure 2—The effect of mast-cell granule protein and proteoglycan on thymidine incorporation of D8 cells.

being a rapid and sustained increase in cell number over the control cultures (two-way ANOVA, $P < 0.01$).

The paradoxic effect of intact mast cells and isolated granules on fibroblasts suggested a tumor-specific release of heparin by mast cells in coculture. This was confirmed by the measurement of release of labeled proteoglycans from mast cells (Table 6). Coculture with tumor cells significantly increased the amount of soluble proteoglycan in the supernatant, while fibroblasts had no significant effect.

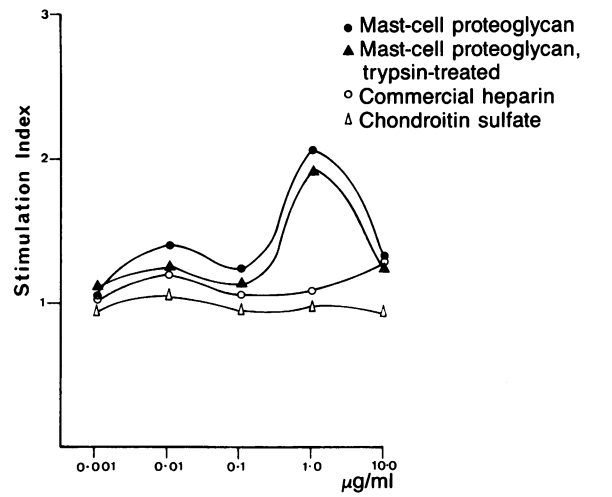

Figure 3—Influence of a variety of glycosaminoglycans on tumor proliferation.

Table 4—The Influence of a Variety of Heparins on the Proliferation of D9 Tumor Cells

Heparin concentration (μg/ml)	Source of heparin			
	Mast cells	CSL	DB	W
0 (Control)	960 ± 75	1178 ± 102	1289 ± 349	1118 ± 57
0.001	1121 ± 138	1339 ± 270	1494 ± 334	1377 ± 123
0.01	915 ± 88	1072 ± 75	1124 ± 24	1137 ± 24
0.1	1024 ± 341	977 ± 97	1261 ± 511	1038 ± 75
1.0	1302 ± 54	1363 ± 740	1170 ± 365	1095 ± 263
5.0	4721 ± 55	1139 ± 125	1428 ± 630	1042 ± 48
10	4547 ± 108	1136 ± 72	1262 ± 101	1112 ± 137
50	4652 ± 313	1449 ± 144	2299 ± 124	2380 ± 169
F (one-way ANOVA, *df* = 7,24)	194	1.218	5.521	10.013
P	<0.01	NS	<0.01	<0.01

Results are expressed as ^{3}H-thymidine uptake mean ± SD.
DB, David Bull Laboratories; W, Weddel Pharmaceuticals.

Discussion

Within 2 years of his identification of the mast cell, Ehrlich noted its presence in tumors.[21] These findings were confirmed, and further extended, by the observation that degranulation and stromal metachromasia occur at the sites of active tumor growth.[22] Equally, in experimental animals, the mast-cell reaction to a tumor corresponded to its proliferative capacity,[23] and stromal metachromasia corresponds to areas of infiltrative growth of human tumors.[24,25]

The studies described herein provide a rational basis for these findings, with the demonstration of the mast cell as the sole nonlymphoid inflammatory cell capable of enhancing tumor growth. The remarkable degree of stimulation of spontaneous tumors (highest stimulation index, 29) is most pertinent in view of the criticism made of the frequent use of carcinogen-induced tumors in the study of host/tumor relationships.[26] The sarcomas D8 and D9 however, were used in most experiments because of their greater morphologic and functional stability; on prolonged culture the carcinomas tended to lose their epithelial morphology, perhaps because of fibroblast overgrowth.

Previous studies from this laboratory[27] and elsewhere[28,29] have demonstrated mast-cell- or basophil-mediated tumor cytotoxicity *in vitro*. However, this was only seen with mast cell/tumor ratios >20:1,[27] with the addition of exogenous H_2O_2 and iodide,[29] or with the induction of basophil degranulation, and even then only 10% of the tumor cells were damaged.[28] The mast cell/tumor ratios used in the author's experiments (10:1–1:100) were chosen arbitrarily but are thought to represent a more realistic model for mast cell/tumor interactions *in vivo*.

Although isolated mast-cell granules were equally mitogenic for both tumor cells and fibroblasts, the mitogenic capacity of intact mast cells was tumor-specific. This specificity was confirmed by augmentation of labeled proteoglycan release by tumor cells but not by fibroblasts. When the intact mast cell, granule, and purified heparin dose-response curves for D8 were examined (based on 1 mast cell = 10^3 granules[30] = 0.1

Table 5—The Effect of Heparinase Treatment on the Capacity of Mast-Cell Heparin to Stimulate D9 Tumor Cell Proliferation

Medium	Tumor cell thymidine uptake (cpm, mean ± SD)	
DME + buffer	2,758 ± 321	*
DME + heparinase	3,626 ± 145	
DME + 10 μg/ml mast cell heparin + buffer	15,915 ± 1432	†
DME + 10 μg/ml mast cell heparin + heparinase	3,091 ± 332	

* t = 4.925; df = 6; $P < 0.01$.
† t = 17.453; df = 6; $P < 0.001$.

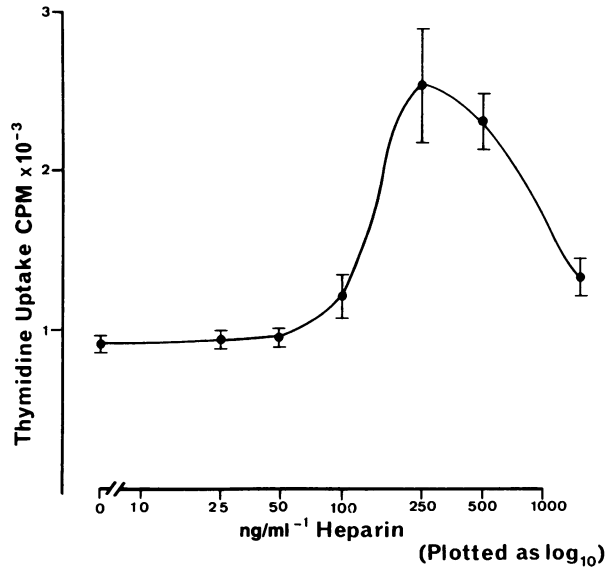

Figure 4—Dose-response profile for purified mast-cell heparin.

ng heparin,[31] the data correlated closely, suggesting a high degree of availability of heparin from the mast cells.

Heparins, in tissue culture, have been shown to both stimulate and inhibit cell growth, depending on both the cell type[32] and the source and structure of the heparin.[33,34] Commercial heparin is a mixture of glycosaminoglycans of molecular weights of 3000 to 37,500 daltons, purified and presented for its anticoagulant activity (for a review see Jaques[35]). As has been demonstrated here, it is not an adequate model for native mast-cell heparin, a proteoglycan of 750,000 daltons molecular weight.[11]

Mast-cell heparin must now be regarded as a growth factor, because it clearly meets at least one definition of positive growth factors,[36] in that it stimulates cell proliferation, in an adequate culture medium, at concentrations likely to be reached *in vivo*. Among the growth factors, it is unique in its proteoglycan structure. The mitogenic activity was due to heparin and not a bound protein, as evidenced by its resistance to trypsin degradation but total abolition by heparinase. However, heparin may have acted as a carrier of a mitogenic protein component of the albeit low serum content of the culture medium. Had this been so, commercial heparins would have been expected to be of equal or greater mitogenic potency. Because commercial heparins were found to be far less active, this hypothesis seems unlikely.

The release of heparin from mast cells by tumors may have other sequelae which might also be favorable to tumor growth. Mast-cell heparin has been shown to stimulate capillary endothelial migration,[37] and commercial heparin acts as a cloning agent for human endothelial cells in the presence of high serum concentrations and endothelial cell growth factor.[38] Mast-cell heparin has potency as an endothelial mitogen in the absence of specific endothelial growth factors, a potency not shared by commercial heparin (unpublished observations). Thus, the direct growth promotion of tumor cells may be accompanied simultaneously by the provision of a blood supply.

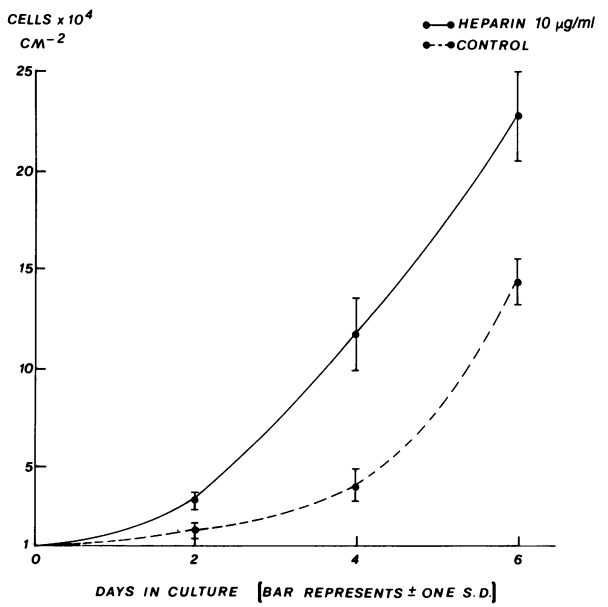

Figure 5—Stimulation of growth of D9 tumor cells by mast-cell heparin.

Table 6—$^{35}SO_4$-Labeled Proteoglycan Release From Mast Cells in Culture

Cells in culture	dpm (mean ± SD)	
10^4 mast cells alone	82 ± 17	] †
10^4 mast cells + 10^5 fibroblasts	61 ± 36	]*
10^4 mast cells + 10^5 D8 tumor cells	185 ± 45	]

* $t = 4.332$; $df = 6$; $P < 0.01$.
† $t = 4.257$; $df = 6$; $P < 0.01$.

Mast-cell heparin has also been recently shown to be a potent suppressor of lymphocyte activation[39] and thereby may further facilitate neoplastic proliferation by the inhibition of putative antitumor responses.

Although in these experiments the expression of this activity was exclusive to tumor/mast cell cocultures, the relevance of the mitogenic activity may not be confined to neoplasia; fibroblasts phagocytose granules after mast-cell degranulation,[10] and this may be a mode of initiating proliferation and tissue repair. Connective-tissue proliferation after mast-cell degranulation has been previously ascribed to histamine,[40] but removal of dialyzable mediators from the granules did not reduce their mitogenic capacity in my experiments (Table 2). The macrophage has also been identified as a source of a mesenchymal growth factor.[41] Interestingly, the stimulation index for fibroblasts exposed to mast-cell granules was almost identical to that for the macrophage-derived factor, assayed by ^{3}H-thymidine uptake of 3T3 cells.[42]

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