TumorBiology

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Influence of Mast Cells on Two Murine Mammary Adenocarcinomas

Abstract

A high content of mast cells (MC) is considered characteristic of neoplasias. Some researchers postulate MC as enhancers of tumor development, others as inhibitors. The purpose of this study was to evaluate the ability of peritoneal cavity MC to modulate the in vivo and in vitro growth of two murine mammary adenocarcinomas with low (M3) and high (MM3) metastatic capacity. MC from the peritoneal cavity of normal (NMC) or tumor-bearing mice (TMC) were used. TMC, which by histochemical methods appeared degranulated, were not able to modify the tumorigenicity of both tumors. NMC, in contrast, decreased M3 tumor incidence and cell proliferation in vitro and increased the latency period of only MM3 tumors. No changes in the number of spontaneous lung metastases could be seen in experiments carried out either with NMC or TMC. We conclude that NMC, which are rich in chemical mediators, can modulate some of the first steps of tumor development. Once tumor-mediated degranulation occurs, MC become unable to regulate it.

Introduction

Mast cells (MC), which are located mainly in the periphery of lymphatic and blood vessels, increase in number in several pathologies, such as hypersensitivity, inflammatory responses, peritoneal adhesion formation, sclerodermas [1–4] and, as Ehrlich [5] and others [6–10] found just 1 year after their discovery, in malignant tumors. Their main action in many diseases is well defined, but their role in neoplasias is not clearly elucidated yet. This is partly due to the great number of chemical mediators (heparin or chondroitin sulfate D/E [11], serotonin [12], proteases, chemotactic factors for eosinophils and neu-

trophils, prostaglandins [13]. cytokines, histamine and leukotriene C4 [14]), which are present in MC cytoplasmic granules. When immunologic or nonimmunologic mechanisms trigger MC exocytosis, these substances are liberated affecting at the same time the function of neighboring cells, as well as the extracellular matrix. In neoplasia, invasion and metastases are dependent on the level of cell proliferation, extracellular matrix degradation and microvascularization. Mast cells may have a role in all these processes by enhancing angiogenesis [15, 16], modifying invasion through the release or activation of proteolytic enzymes [17–19] or affecting tumor growth. With respect to the latter point, some studies showed that MC enhance tumor growth [20, 21], while others demonstrated an inhibitory effect [22, 23]. The different effects of MC on tumor development can perhaps be explained by the different types of tumors and the different experimental procedures used.

In the present work, we examined the action of MC on two murine mammary adenocarcinomas, one with low (M3) and the other with high (MM3) metastatic capacity.

Materials and Methods

Chemicals

Pronase was purchased from Serva (Heidelberg, Germany); Eagle's minimum essential medium (MEM; 410–1,500), glutamine and trypan blue from Gibco (Grand Island, N.Y., USA); fetal calf serum (FCS) from GEN (Buenos Aires, Argentina); alcian blue 8GS standard, from Fluka; safranin O, basic fuchsin, indigo carmine, picric acid, Tris buffer, albumin, EDTA, DNase and metrizamide from Sigma (St. Louis, Mo., USA); 24-well plastic dishes from Falcon (Lincoln Park, N.J., USA), and ^{51}Cr-sodium chromate from New England Nuclear, Boston, Mass., USA.

Animals and Tumors

Male Balb/c mice, 9 weeks old, were obtained from the 'A.H. Roffo Institute Breeding Center' (Buenos Aires, Argentina).

Two Balb/c mammary adenocarcinomas (M3 and MM3) with lung metastases were used [24]. They were maintained by grafts of subcutaneous tumor fragments (2 mm in diameter). M3 is a Balb/c spontaneous mammary tumor which kills mice within 40 days. It has a 40% incidence of lung metastasis formation and a latency period (LP) of 6–8 days. The MM3 variant was obtained after serial subcutaneous trochar transplants of M3 pulmonary metastases into the flanks of syngeneic mice. Once MM3 achieved stable growth and metastatic behavior, it was then further maintained by subcutaneous grafts of tumor fragments. This variant showed a 90% metastatic incidence, and killed mice within 60 days, with a LP of 12–14 days.

Preparation of Tumor Cell Suspensions

When tumor-bearing mice reached two thirds of the survival time (24 days for M3 and 42 days for MM3 after subcutaneous transplantation), the tumors were excised under sterile conditions and minced after removal of necrotic tissue. The fragments were enzymatically disaggregated [30] by incubating them in a 0.01% pronase and 0.0035% DNase solution during first 15 min, then 30 min at room temperature. The cells were washed twice in complete medium (CM: MEM plus 2 mM L-glutamine and 80 µg/ml gentamicin) and resuspended in CM supplemented with 10% FCS. Viability, as assayed by the trypan blue exclusion test, was >80%.

Preparation of MC Suspensions

Peritoneal exudate cells of 15 normal and 15 tumor-bearing mice were obtained by washing the peritoneal cavity with 3 ml of Tris-albumin-EDTA buffer, pH 7.6 [25]. Cell pellets from both groups of animals were independently concentrated up to 1.5 ml. MC from normal mice (NMC) and from tumor-bearing mice (TMC) were purified by centrifugation using 24% metrizamide. Cells from the bottom were washed twice in CM. Viability (>95%) was assessed by the trypan blue exclusion test, and purity (98%) was determined using cell preparations obtained by cytocentrifugation. The slides were fixed in Newcomer's solution and stained with 0.5% toluidine blue.

In vivo Assays

Tumorigenicity and Spontaneous Metastatic Ability. Six groups of 10 Balb/c mice each received subcutaneous injections of 3×10^5 M3 or MM3 cells in 100 µl of CM without (control) or with 3×10^4 NMC or TMC (experimental condition) in the right flank.

Local Tumor Growth. Palpations of tumor transplants were done daily in order to determine the LP

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and tumor incidence (TI). LP was defined as the time between the subcutaneous injection of cells and the external palpation of tumors.

TI was defined as the ratio of the number of animals in which tumors reached a diameter of 5 mm and continued to grow, versus the total number of animals. To evaluate tumor growth, the largest perpendicular diameters (L and l) were recorded three times a week, and the volumes were computed by the formula for prolate spheroids, i.e. $V = \pi/6(Ll)^{3/2}$. For each animal, the tumor growth rate was calculated by the linear regression of the ln of the tumor volume during the exponential growth period, and the means of slopes for each group were also calculated.

On days 30 and 40, respectively, after M3 or MM3 cell inoculation, animals were sacrificed under anesthesia. The lungs were removed and fixed in Bouin's solution overnight. The number of metastatic foci was counted under a dissecting microscope.

Tumors together with the adjacent tissues were excised and processed for histological examination.

Histology. Tumors from animals of each group were fixed 12 h in Newcomer's fixative. After three butylic alcohol baths (3 h each) they were embedded in paraffin. This method is suitable to preserve the stainability of MC granules.

To evaluate MC degranulation, consecutive sections (5 µm) were examined after staining with: (a) alcian blue in a staining sequence with safranin (AB-S) [26]. MC stain blue (AB+) if they are activated and/or degranulated, or red (S+) when they are not. (b) Alcian blue in $MgCl_2$, 0.9 M (AB-CEC) [27]: at this critical electrolyte concentration only those MC granules which are rich in heparin stain blue.

To evaluate MC spontaneous proliferation, three nonserial sections were stained according to Gallego's method [28], a topographic technique, in which an acid solution (pH = 3) of basic fuchsin stains MC, and picro-indigo-carmine solution enables to recognize all the tissues.

The MC numbers were registered in 10 randomly selected high power fields (0.25 mm^2) in dermis, hypodermis and peritumoral tissues in experimental animals on day 14. The data obtained were compared with those from mice injected with a normal syngeneic kidney cell suspension (control animals).

In vitro Assays

Evaluation of Tumor Cell Proliferation. M3 or MM3 tumor cells were plated out at 5×10^4 cells ml^{-1} in 24-well cluster dishes in CM supplemented with 10% FCS, and incubated at 37°C for 24 h in a humidified atmosphere of 5% CO_2. The medium was then discharged and the cells washed three times in phosphate-buffered saline, with 10-min intervals between each wash. Then CM was added to the cells, either with only 2% inactivated FCS (control), or with the addition of NMC or TMC, the ratio being MC:TC = 1:10, 1:5, 1:2 and 1:1. The assays were made in quadruplicate; they were reproducible and repeated at least three times.

Additionally, histological studies were performed on coverslips inserted into the culture chambers. In vitro tumor growth was evaluated by determining the protein content in each well [29].

Cytotoxicity Assay

MC-mediated cytotoxicity against M3 cells was assayed. The M3 cells were suspended at a concentration of 1×10^4 cells/ml in CM plus 2% heat-inactivated FCS. Aliquots of 0.2 ml of this suspension were labeled with 20 µCi of ^{51}Cr-sodium chromate during 1 h at 37°C. Thereafter, the M3 cells were washed and resuspended at a concentration of 1×10^5 cells/ml. The cytotoxic assays were performed in 96-well flat-bottom microtiter plates by incubating 1×10^4 M3 cells with mast cells in an effector:target cell ratio of 0.1, 0.2, 0.5 and 1.0 in a final volume of 0.15 ml for 4 h at 37°C in a 5% CO_2-95% humidified incubator. Then the radioactivity of the supernatants and of the residual cells was measured. The mean release of ^{51}Cr in quadruplicate samples was expressed as percentage of the total radioactivity.

Statistical Analysis

Latency and TI were evaluated by the χ^2 test, tumor growth and MC numbers in peritumoral tissues by ANOVA, data from tumor metastases by the Mann-Whitney rank sum test, and data of the cell proliferation in vitro were analyzed by Student's t test. Statistically significant differences were assumed when $p \leqslant 0.05$ or better.

Results

In order to evaluate the tumor development and MC distribution, we examined histologically preparations obtained from the tumor cell inoculation site. In those animals where tumors developed, both adenocarcinomas grew in a spheroidal shape and were adherent to the muscle underlying the skin
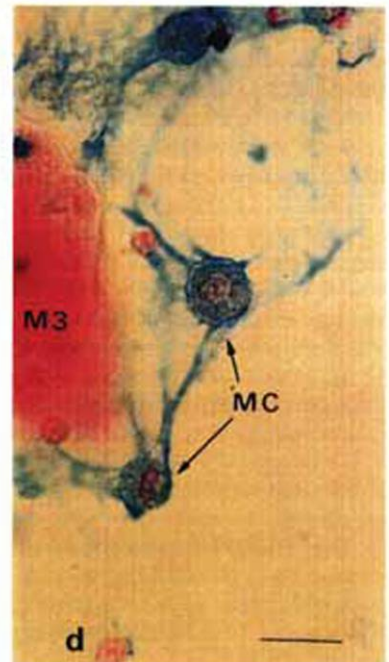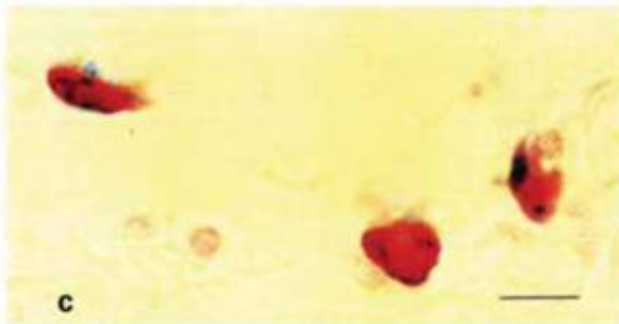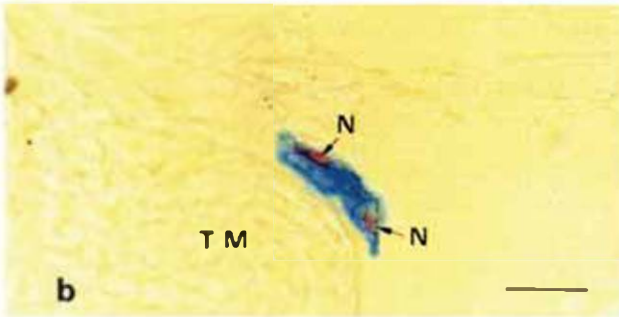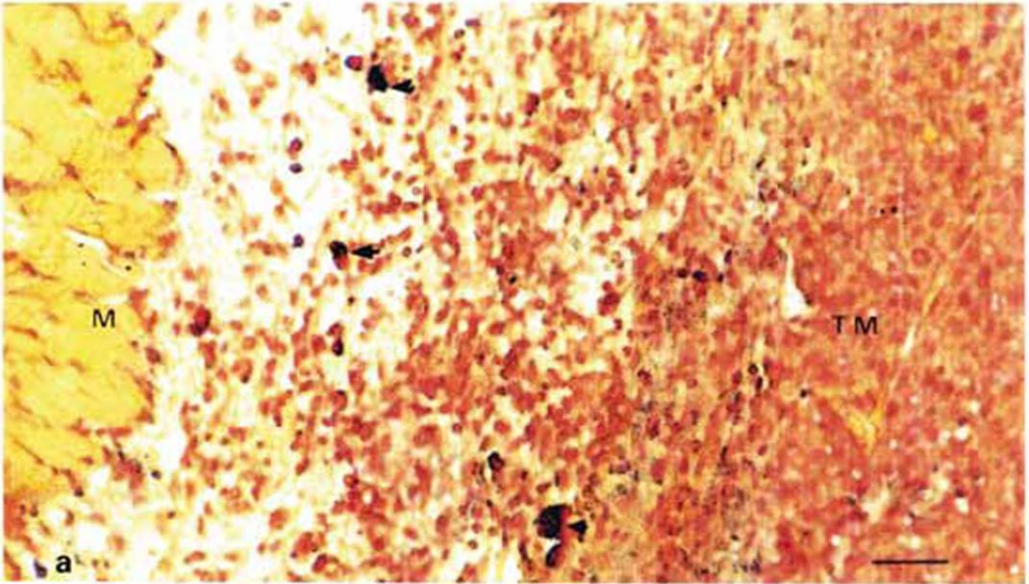
Fig. 1. a Transverse section of the integument showing the tumoral mass (TM) under muscle (M). Arrowheads show MC in the peritumoral tissue. Gallego's staining. Bar = 50 μm. **b** Two peritumoral MC stained AB+. N = Nucleus; TM = tumoral mass. AB-S method. Bar = 15 μm. **c** MC near the epidermis stained partially S+ (>70%) and AB+. AB-S method. Bar = 15 μm. **d** MC cocultured with M3 cells for 24 h. A similar image is observed with MM3 cells. MC stained AB+ by tumor action. AB-S method. Bar = 15 μm.

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(fig. 1a). When both tumors were tested in their ability to attract MC, no statistically significant differences in MC numbers were detected, neither in the dermis, or hypodermis, nor in the peritumoral tissue between both tumors. However, MC numbers found in tumors were always significantly higher than in control animals (fig. 2).

Tumor-activated MC lose their mediators during the degranulation process. To evaluate whether M3 or MM3 have the same effect on triggering MC exocytosis, tissue specimens were stained with the AB-S method. We found that MC near or inside the tumor mass were AB+, which meant that they were activated/degranulated (fig. 1b). In contrast, MC distant from the tumors or derived from animals in which tumors did not develop, stained red with safranin (S+), i.e. they were not affected (fig. 1c). MC from regions between epidermis and peritumoral tissues stained partially AB+ and S+ indicating that they were partially degranulated. No significant differences in MC numbers of each phenotype were observed in control or experimental tissue specimens either of M3 or of MM3 tumor-bearing mice.

A comparison of consecutive AB-CEC-stained sections to those stained with AB-S revealed that only S+ MC were AB-CEC+, so they were rich in heparin, while AB+ MC had lost this proteoglycan, and probably other chemical mediators not tested in this work.

It is known that both connective tissue and peritoneal cavity MC from normal animals are S+ and rich in heparin. Employing the above-mentioned staining methods, we found that 70% of peritoneal cavity MC from tumor-bearing animals were AB+ and AB-CEC-, like those of peritumoral tissues.

In vivo Studies

To evaluate the influence of MC in tumor development, TC were coinjected with NMC
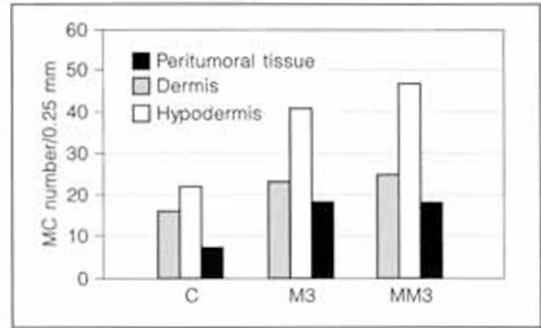

Fig. 2. The mean of MC number in kidney (C), M3- and MM3-implanted mice, 14 days after transplantation. ANOVA, p < 0.01 vs. control. SD was usually less than 10% of the mean value.

or TMC. As shown in table 1 when the MM3 cells were coinjected with NMC, the LP was significantly increased, but TI was not reduced in a significant way. On the contrary, NMC diminished M3 TI as much as 70%, but no differences were detected in the LP in those animals in which tumor developed (30%). MC did not modify the number of M3- or MM3-induced lung metastases (table 2). Nevertheless, it was interesting to observe that NMC diminished MM3 TI and the number of lung metastases, although being nonsignificant (p = 0.085 vs. control, Mann-Whitney rank sum test).

In animals inoculated with TC which developed tumors despite the presence of MC, the slope of the exponential tumor growth curve resembled that of the controls, as calculated from the ln of tumor volume during 20 days after the tumor became palpable.

In vitro Studies

Since NMC decreased the M3 tumor incidence in vivo, we studied the action of NMC on M3 and MM3 tumors in vitro. Indeed, we found that they inhibited the proliferation of M3. The proliferation rate was found to be

	Control[a]	Treatment[b]	
		NMC	TMC
M3 LP	8/10	3/3	10/10
MM3 LP	10/10	5/10*	10/10
M3 TI	10/10	3/10*	10/10
MM3 TI	10/10	8/10	10/10
M3 growth rate[c]	0.209 ± 0.02	0.210 ± 0.02	0.220 ± 0.02
MM3 growth rate[c]	0.163 ± 0.02	0.175 ± 0.02	0.167 ± 0.02

Data corresponding to one experiment. Similar results were obtained in a second assay. Latency was expressed as a number of animals with tumor at 7 days for M3 and 16 days for MM3 cells vs. total of animals in which tumor developed. * $p < 0.05$ vs. control and M3-TMC (χ^2 test).

[a] Mice injected with 3×10^5 TC.
[b] Mice coinjected with 3×10^5 TC and 3×10^4 NMC or TMC.
[c] Means $\pm$ SD of growth slopes. n = 10.

Table 2. Number of M3 and MM3 lung metastases

		Control[a]	Treatment[b]	
			NMC	TMC
M3	Median	0	0	0
	Range	0–2	0–1	0–1
MM3	Median	2.5	0	0
	Range	0–29	0–2	0–10

Data corresponding to the experiment shown in table 1.
[a] Mice injected with 3×10^5 TC.
[b] Mice coinjected with 3×10^5 TC and 3×10^4 NMC or TMC.

dependent on the NMC concentration (fig. 3). On the contrary, NMC did not affect the MM3 cell proliferation rate.

Since one explanation for the inhibition of tumor proliferation was a possible cytotoxic effect, we investigated cytotoxicity of MC on M3 cells, but we could not detect any tumorici-dal activity at any MC:TC ratio, since spontaneous ^{51}Cr release was always lower than 20%.

In order to observe a possible direct action of TC on NMC, coverslips were inserted into culture chambers and stained 24 h after having been seeded with AB-S and AB-CEC to test MC activation and heparin content.

Despite the fact that NMC added to wells containing both tumor cells were S+ at 24 h, they all turned into AB+ and were AB-CEC–. This observation was consistent with what was observed in specimens from animals of in vivo experiments.

Discussion

Since an association between MC and neoplasia and MC degranulation in the vicinity of tumors has been verified, several studies were carried out to determine the functional role of MC. In the present investigation, we have tested their ability to modulate the function of two murine mammary adenocarcinomas with different metastatic indices.

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These tumors, as other neoplasias, were able to recruit MC from the peritumoral tissue. This quality has been correlated to grades of tumor malignancy and mitotic activity [30], and was considered to be a prognostic marker in soft tissue sarcomas [31]. We observed that M3 and MM3 cells induced MC proliferation to the same extent. Interestingly, in our experience [32], a murine sarcoma with a higher angiogenic potential, S13, induced a greater MC proliferation. It is well known that MC increase tumor angiogenesis [33], but it could be thought that tumor-mediated MC recruitment could be in part a consequence of tumor angiogenicity.

MC degranulation was similarly elicited by both tumors, and a subsequent change in their phenotype (from S+ to AB+) was detected. Furthermore, similar results were obtained when the same staining methods were employed on NMC cocultured with TC in vitro, or with TC-conditioned medium (data not shown). Taken together, these observations suggest a direct action of TC on MC, TC probably releasing soluble factors which induce MC exocytosis. Furthermore, we observed that they also induced a systemic action since as much as 70% of TMC were activated.

Some reports on murine tumor models [34, 35] and different types of human cancer [31, 36, 37] showed that MC inhibited tumor growth, while other experimental studies demonstrated an opposite effect in vivo and in vitro [38, 39].

Our in vivo assays indicated that MC did not modify the growth rate of the tumors studied. However, NMC caused a decrease of 70% in M3 TI, while no effect was observed when MM3 cells were used. In vitro experiments corroborated the in vivo results, since NMC inhibited only the M3 monolayer growth in a MC:TC-dependent way, without affecting MM3 development. TMC lacking chemical mediators were not able to modulate it.
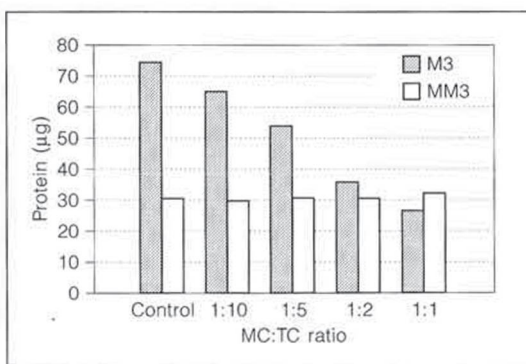

Fig. 3. Effect of MC on the proliferation of M3 and MM3 cells, in the presence of 2% inactivated FCS. The same number of TC (5×10^4) was seeded in each well in both experiments. MM3 has less adhesion, cellular size and doubling population time. $p < 0.01$ vs. control; Student's t test. n = 4. SD was usually less than 10% of the mean value.

We have previously demonstrated that these murine mammary adenocarcinomas express heparin receptors [40]. In addition, this glycosaminoglycan inhibited their in vitro growth [41].

The resistance of MM3 to heparin-rich NMC is still unexplained since we observed [29] that when heparin was added in vitro to the CM, MM3 cell proliferation was inhibited. In those earlier experiments, heparin was effective at a concentration near 200 μg/ml, but the MC heparin concentration is certainly too low to diminish MM3 cell growth [42]. Furthermore, as far as a MC action is concerned, it is most likely that other MC chemical mediators, besides heparin, may inhibit M3 cell proliferation.

Since the M3 tumor incidence was actually inhibited by NMC, which are the differences between M3 and MM3 tumors which lead them to different responses? As MM3 is a tumor derived from M3 lung metastases, it implies cellular differences which were cytogenetically demonstrated [24]. These differ-

ences probably allow MM3 cells to escape from host defense mechanisms.

Additionally, some reports have indicated that MC granules enhanced the proliferation of a cellular clone derived in a similar manner to MM3 [38], and of a murine sarcoma [39]. Based on our and the above-mentioned observations, we conclude that the action of MC is not only a consequence of a MC degranulation but also of the type of TC on which MC mediators react.

Considering that NMC had only diminished the M3 TI by probably inhibiting cell proliferation, and increased MM3 LP in vivo,

here affecting neither the tumor growth index nor lung metastasis formation, we suggest that MC could modulate early stages of tumor progression, thus inhibiting its subsequent development. However, if tumor proliferation is not stopped at this point, it will continue its exponential growth.

Acknowledgment

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