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GM-CSF restoration of a differentiated (growth factor-regulated) phenotype in an anaplastic tumor

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Summary. GM-CSF (granulocyte-macrophage-derived colony-stimulating factor) is a differentiation agent that stimulates bone marrow activity in patients receiving chemotherapy. GM-CSF (1 μg/ml daily for 10 days), administered intralesionally, was evaluated to determine whether it would restore a more differentiated phenotype to an anaplastic, rapidly growing, hormone-independent variant (R3327 MAT-LyLu) of the Dunning prostatic adenocarcinoma. Immunohistology was used to quantitate the expression of epithelial growth factor receptors (rEGF) and the tissue testosterone content. GM-CSF therapy significantly ($P < 0.05$) restored rEGF expression and tissue testosterone to levels associated with better differentiated, slower growing, androgen-dependent Dunning variants (R3327 H and G). GM-CSF may have a role in treatment of prostatic cancers by promoting androgen and epithelial growth factor regulation.

Key words: GM-CSF – Prostate – Dunning tumor – Therapy – Differentiation – Growth factors

Myeloid production of blood cells is regulated by four cell-derived macromolecules, which act upon progenitor cells to form granulocytes and macrophages [13]. These glycoproteins are identified by their ability to stimulate colony formation in both in vitro and in vivo assay systems [1, 16]. Each of these colony-stimulating factors is coded by an individual gene having no sequence homology and acts via interaction with a relatively low number (a few hundred/cell) of very specific membrane receptors. Of importance to this study is the observation that the corresponding human and murine colony-stimulating factors share homology, but are not always cross-species-reactive [12].

Cells known to produce colony-stimulating factors include T-lymphocytes, monocytes, fibroblasts, and both endothelial and epithelial cells [11]. The several activities described for these colony-stimulating factors [12] include the ability to commit the differentiation of bipotential progenitor cells irreversibly to either granulocytes or macrophages [14].

Several of these factors are now available as recombinant proteins. One of these, GM-CSF (granulocyte-macrophage-derived colony-stimulating factor), has been shown in both primates and humans to effectively elevate neutrophile levels following chemotherapy or bone marrow transplantation [3–5, 10, 15].

In this study, the effect of recombinant GM-CSF upon restoration of growth regulation (mediated via androgen and/or epithelial growth factor) was assessed in an anaplastic prostatic tumor. Comparisons were made between GM-CSF-treated and non-GM-CSF-treated rats bearing the hormone-insensitive, anaplastic, rapidly growing Dunning R3327 MAT-LyLu tumor, as well as with the Dunning R3327 H and G sublines, which are better differentiated, more slowly growing and hormonally sensitive (Table 1).

Materials and methods

Dunning tumor model

The Dunning R3327 prostatic adenocarcinoma is carried in male Copenhagen X Fischer F1 rats. The R3327 H subline is a well-

Table 1. Comparisons between Dunning R3327 sublines

Tumor subline	Histological characterization	Doubling time (days)
R3327-H	Well-differentiated Slow-growing Hormone-sensitive	15–20
R3327-G	Poorly differentiated Fast-growing Hormone-sensitive	2
R3327-MAT-LyLu	Anaplastic Rapid-growing Hormone-insensitive Metastatic	1.2

Table 2. Distribution of experimental animals

Group	No. of rats	Tumor subline	Treatment
1	5	R3327-H	None
2	10	R3327-G	None
3	14	R3327-MAT-LyLu	RPMI 1640
4	20	R3327-MAT-LyLu	GM-CSF/RPMI 1640

Table 3. Effect of GM-CSF on growth of the Dunning MAT-LyLu tumor

Day	Tumor size (cm^2)		
	Control	GM-CSF treatment[a] and sacrificed on day 11	GM-CSF treatment[b] and sacrificed on day 15
1[a]	1.8 ± 0.3	1.7 ± 0.3	1.8 ± 0.4
4	4.6 ± 1.1	4.7 ± 0.9	4.9 ± 1.1
8	7.5 ± 1.9	6.2 ± 0.9	6.8 ± 1.0
11	11.0 ± 1.2	9.9 ± 1.3	10.7 ± 0.9
15	16.4 ± 1.5		15.2 ± 1.3

[a] GM-CSF treatment began on day 1 and continued daily for 10 days

[b] Day 1 denotes initial tumor size prior to initiation of 10 days of GM-CSF therapy

differentiated prostatic adenocarcinoma, possessing androgen-sensitive columnar epithelial cells and numerous well-differentiated acini containing both prostate-specific acid phophatase and secretory material. The tumor is slow growing, hormonally sensitive, and will involute upon castration or estrogen administration [9]. An androgen-insensitive cell population is also present and accounts for tumor relapse following hormonal manipulations. The R3327 G subline is a much more rapidly growing and undifferentiated carcinoma composed of densely packed primitive cells in varying stages of disarray. This is an androgen-sensitive variant; however, unlike the R3327 H tumor, it expresses neither involution nor cessation of growth following castration [7]. An additional tumor has been derived termed the R3327 AT subline. This is a rapidly growing, anaplastic, hormone-insensitive variant, which grows equally well in females and in intact or castrated males. This R3327 AT tumor gave rise to an additional subline which exhibits a high incidence and predictable pattern of metastatic dissemination. The R3327 MAT-LyLu tumor (Metastatic AT LYmph nodes, LUng) metastasizes to the lymph nodes and lungs [8]. Comparisons between the Dunning R3327 sublines are listed in Table 1.

Implantation of tumors

Tumors were maintained in Copenhagen X Fischer F1 rats by S.C. injection into the left flank of 10^6 viable tumor cells obtained by collagenase digestion. Viability was determined by exclusion of 0.4% trypan blue dye. At the time of tumor implantation animals were sexually mature and weighed between 200 and 300 g. Tumor progression was determined at least once a week using vernier microcalipers.

Experimental design

A total of 49 rats were utilized; they were distirbuted into four groups as shown in Table 2.

Therapy

Animals which received recombinant murine GM-CSF (Immunex; Seattle, Wash.) were inoculated intratumorally for ten consecutive days with 1 μg/ml (specific activity in excess of 4×10^7 units/mg protein) suspended in a 1-ml volume of RPMI 1640. Non-GM-CSF-treated rats bearing the R3327 MAT-LyLu tumor received similar daily inoculations of RPMI 1640. In order to determine the duration of any effect of GM-CSF following the cessation of daily treatment, rats bearing R3327 MAT-LyLu tumors and treated with GM-CSF (group 4) were initially divided into two subgroups: (4a) those sacrificed 1 day after cessation of therapy (day 11); and (4b) those sacrificed 5 days after cessation of therapy (day 15). Tumors were measured with vernier calipers 1, 4, 8, 11, and 15 days after initiation of therapy (Table 1). Rats bearing non-GM-CSF-treated Dunning R3327 H, G or MAT-LyLu subline tumors were sacrificed when tumors were comparable in size to those in the GM-CSF-treated groups.

Immunohistochemistry

At the time of sacrifice tumors were removed, frozen at −80 °C and subjected to immunohistologic characterization using the method of

Table 4. Mean intensity of reactivity of the various dunning sublines following GM-CSF treatment

	Experimental groups				
	H-subline	G-subline	MAT-LyLu subline		
			Non-GM-CSF-treated	GM-CSF treated	Significance[a]
No. of animals	5	10	14	20	
Growth factors					
rEGF	2.0 ± 0.7	1.7 ± 0.5	1.1 ± 0.7	2.5 ± 0.9	$P < 0.05$
Testosterone	2.8 + 0.5	2.7 ± 0.7	1.0 ± 0.9	2.3 ± 0.9	$P < 0.05$

[a] MAT-LyLu subline: non-GM-CSF-treated vs GM-CSF-treated

Table 5. Results of GM-CSF therapy of rats bearing Dunning R3327 MAT-LyLu tumor assessed 1 and 5 days after cessation of treatment

Non-GM-CSF-treated (group 3)	rEGF	Testosterone
1	2	1
2	1	0
3	2	1
4	1	1
5	2	1
6	0	0
7	1	2
8	1	1
9	1	2
10	1	0
11	0	3
12	2	1
13	1	1
14	1	0
	1.1 ± 0.7	$1.1 + 0.9$
GM-CSF-treated (sacrificed on day 11) (group 4a)		
1	2	2
2	3	2
3	3	2
4	4	2
5	1	2
6	3	4
7	2	3
8	2	1
9	3	1
10	2	2
	2.5 ± 0.9	2.1 ± 0.9
GM-CSF-treated (sacrificed on day 15) (group 4b)		
11	3	1
12	2	2
13	3	3
14	1	2
15	3	2
16	3	2
17	3	3
18	2	3
19	4	3
20	1	4
	2.5 ± 1.0	2.5 ± 0.9

Hsu [6]. Briefly, 5-μm frozen sections prepared using a cryostat, were applied to glass slides, dipped in acetone for 10 s and allowed to air dry for 1 h. Slides were then stored at −20°C prior to use and rehydrated in PBS for 5 min before the addition of non-specific serum (2%) of goat origin. Primary monoclonal antibodies [against the receptor for epithelial growth factor (rEGF; Biogenex) and against testosterone (Biogenex)] were added and left for 2 h at room temperature. Slides were washed with PBS, and the link antibody (biotinylated rabbit antimouse immunoglobulin) applied for 20 min. Following more washes in PBS the substrate 3-amino-9-ethylcarbazole (AEC) was added, allowed to incubate for 10 min, washed in distilled water, and counterstained with hematoxylin. Three to ten slides (with a mean of approximately four) from each specimen were examined and graded for intensity of reactivity, which was quantitated on a scale of 0 to 5 (5 being most intense). Negative control slides on which the primary antibody was substituted with non-specific goat serum were run for each specimen. In order to determine intra- and interobserver differences, slides were evaluated blind by each author and by a Board Certified Pathologist. Differences encountered between independent evaluations and observers were minimal.

Statistical analysis

Mean and standard deviations were determined for reactivity to each antiserum. Significant differences were determined using Student's *t*-test. The effect of GM-CSF on tumor growth was determined using a *t*-test for independent sample comparisons.

Results

The results of GM-CSF treatment upon growth of the Dunning R3327 MAT-LyLu tumor are seen in Table 3. No significant differences in growth were recorded on any day, although on day 8 a *T*-value of 2.00 showed near-significant results for the GM-CSF treated animals sacrificed 1 day after the cessation of therapy.

Immunohistochemical analysis was performed on all tumor groups for quantitation of the levels of rEGF and tissue testosterone (Table 4). There were no apparent differences (Table 5) in the immunoreactivity between GM-CSF-treated Dunning R3327 MAT-LyLu tumors obtained 1 or 5 days after cessation of therapy and final results obtained from groups 4a and 4b were pooled for a sample size of twenty. Data from rats bearing Dunning R3327 H and G are not shown in Table 5, because GM-CSF was not administered to these animals.

Table 4 demonstrates that phenotypic expression of rEGF and tissue levels of testosterone decrease as one progresses from relatively slow-growing, better differentiated, hormone-dependent tumors in the direction of those that are more aggressive (metastatic), anaplastic, rapidly growing and non-hormone-dependent. More specifically, GM-CSF treatment resulted in an increased rEGF intensity of reactivity (2.5 ± 0.9 vs 1.1 ± 0.7, $P < 0.05$) in the MAT-LyLu subline that exceeded the intensity in either the H (2.0 ± 0.7) or the G (1.7 ± 0.5) subline. The rEGF pattern of reactivity was membranous in nature and found throughout all groups. Similarly, the GM-CSF treatment resulted in an increased intensity of testosterone reactivity (2.3 ± 0.9 vs 1.0 ± 0.9, $P < 0.05$) in the MAT-LyLu subline that was slightly less than the intensity of both the H (2.8 ± 0.5) and G (2.7 ± 0.7) sublines. We observed no alterations in histological or cellular patterns as a result of GM-CSF treatment other than those identified through immunohistological staining.

Discussion

Epithelial growth factor and testosterone [2] are associated with prostatic growth, morphogenesis and differentiated function. Following GM-CSF treatment rEGF and testosterone immunohistochemical reactivity increased significantly ($P < 0.05$) in an anaplastic prostate tumor (1.1 ± 0.7 to 2.5 ± 0.9 and 1.0 ± 0.9 to 2.3 ± 0.9 respectively) and approximated that of the well-differentiated Dunning R3327-H subline tumor (2.0 ± 0.7 and 2.8 ± 0.5 respectively; Table 4). The findings of this experiment suggest that, in rats bearing the anaplastic Dunning R3327 MAT-LyLu tumor, recombinant GM-CSF promotes the expression of rEGF and the tissue retention of testosterone in this otherwise hormone-insensitive subline.

The restoration of more normal growth regulation following GM-CSF therapy could have implications for the treatment of prostatic cancer. In patients with metastatic prostate cancer, hormone therapy is frequently used to control the disease. However, following a period of regression, the tumor usually re-establishes itself as a more aggressive hormone-independent variant, which is refractory to further hormonal manipulations (relapse to castration). Since it is unlikely that spontaneous mutations would consistently eliminate hormone responsiveness in recurrent prostatic tumors, it is more likely that expression of, or the response to, normal growth-regulatory influences, is suppressed. Hormone-insensitive clones are apparently well represented in heterogeneous prostatic tumors, even at an early stage. It would be of clinical importance if these types of recurrent tumors could either retain or reestablish, once again, a more differentiated phenotype in the androgen-deprived patient. This would permit additional therapeutic growth-regulatory manipulations (i.e., exogenous estrogen) to restrain uncontrolled tumor expansion, and presumably delay the evolution to complete hormonal independence in these already elderly patients. An alternate, "prophylactic," approach could utilize GM-CSF administered prior to hormonal manipulations to differentiate the relatively low (15%) number of hormone-independent cells already present. This might result in an even higher initial response rate than the current 80–85% in previously untreated patients.

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