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Effects of Tumor Necrosis Factor on Pregnancy-dependent Mammary Tumors in GR/A Mice

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ABSTRACT—This experiment was designed to examine the effects of twice daily injections of tumor necrosis factor (TNF; 5 μg/0.1 ml physiological saline) on pregnancy-dependent mammary tumor (PDMT) in GR/A mice which appears during pregnancy, disappears soon after parturition and appears again during the subsequent pregnancies. The significantly positive correlation between the size of PDMT and the period of vehicle treatment observed in the control disappeared by TNF treatment. Furthermore, the linear regression coefficient was apparently higher in the control than in the experimental group. The number of PDMT was also decreased by TNF. Immunohistochemical staining of products of c-*jun* in PDMT was stimulated and those of c-*fos* and N-*myc* were inhibited by TNF. TNF had no effects on reproduction and the weights of anterior pituitary, adrenals and ovaries. All results have demonstrated that TNF inhibits the growth of PDMT corresponding to the period of treatment and its effect may be partly through the modulation of some oncogene expression with little influence on the endocrine parameters.

INTRODUCTION

The GR/A mouse is characterized by the development of pregnancy-dependent mammary tumor (PDMT), which appears after the middle of pregnancy, reaches the maximal size at the end of pregnancy, regresses and disappears soon after parturition regardless of lactation [13, 18]. PDMT appears again during the subsequent pregnancies and its incidence and growth often increase with the additional pregnancies [13, 18]. While estrogen, progesterone and placental lactogen were found to participate in the growth of PDMT [16, 17], the mechanism of the growth and regression of this type of lesion is mostly obscure.

Recently, the contribution of autocrine and paracrine systems to the growth or regression of normal and neoplastic mammary cells has been reported [for review, 9]. Tumor necrosis factor (TNF), which is cytotoxic and cytostatic against several transformed cells, is a protein produced by macrophages and it has antitumor effects on transplantable tumors [2, 5, 11, 12]. TNF has also been reported to be produced in the carcinomas of the breast, ovary and colon [11].

Furthermore, numerous works suggesting the participation of oncogenes and growth factors in normal and neoplastic growth of mammary glands have been reported [for reviews, 3, 6, 8, 15].

The objective of this study is to examine the effects of TNF on the growth of PDMT and the immunohistochemical expression of some oncogenes.

MATERIALS AND METHODS

Animals

GR/AMei mice maintained in our laboratory by strict brother×sister mating were used. At 70–75 days of age, females were placed with males. Pregnant mice were housed individually and placed again with males only near parturition to induce concurrent pregnancy. Only mice that developed PDMT during the 1st pregnancy and delivered concurrently were used.

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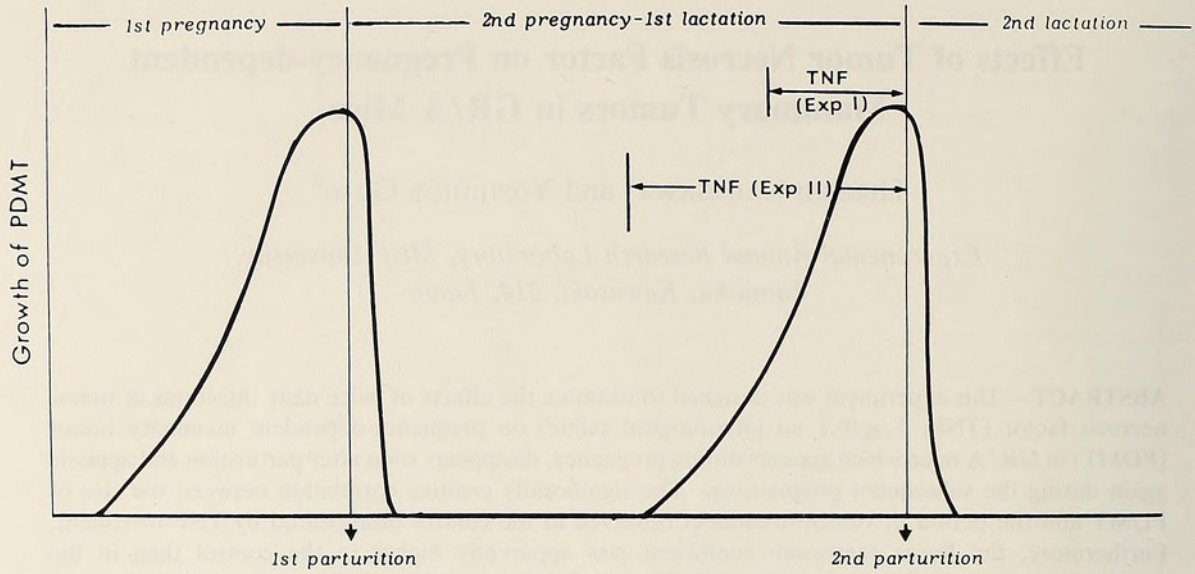


FIG. 1. Experimental schedule. In Experiment I (Exp I), experimental animals received twice daily injections of 5 μg/0.1 ml physiological saline from day 15–18 of the 2nd pregnancy-1st lactation, when PDMT sizes achieved 4–5 mm in diameters, until the day of the 2nd parturition. In Experiment II (Exp II), injection was started on day 10 when PDMT were still unpalpable. Controls were given vehicle only during the corresponding periods.

Throughout the experiment, mice were kept in aluminium cages (12×28×13 cm) with wood shavings, maintained in an animal room, which was air-conditioned (22–24°C and 55–75% relative humidity) and artificially illuminated (14 hours of light from 5:00 AM to 7:00 PM), and provided with a commercial diet (Lab MR Breeder; Nihon Nosan Kogyo KK, Yokohama, Japan) and tap water *ad libitum*.

Treatments (Fig. 1)

In Experiment I (Exp I), animals of which PDMTs achieved 4–5 mm in size, *i.e.* on day 15–18 of the 2nd pregnancy-1st lactation, were given twice daily (8:30 AM and 5:00 PM) intratumoral injections of TNF (Peninsula Lab. Inc., Belmont, CA, USA; 5 μg/0.1 ml physiological saline) until the day of parturition.

In Experiment II (Exp II), beginning day 10 of the 2nd pregnancy-1st lactation, when PDMT was still unpalpable, mice received twice daily subcutaneous injections of TNF (5 μg/0.1 ml) near the places where PDMT appeared at the 1st pregnancy. After PDMT became palpable, the treatment was changed to the intratumorous injection and continued until the day of the 2nd parturition. TNF solution was stored at −20°C until used.

Control mice were given the vehicle only during the corresponding periods. However, the data were pooled in the Results section, since no difference was observed in all parameters examined.

Some experimental and control mice of Experiment II were killed by decapitation under the light ether anesthesia at the end of the 2nd pregnancy and PDMTs were immediatly removed, frozen at −80°C and used for immunohistochemical staining.

The remaining mice in each group were subjected to check the reproduction.

Size and number of PDMT

Each mouse was checked daily for palpable PDMT from day 12 of the 1st and the 2nd pregnancies until parturitions. The size of the first PDMT expressed in terms of the geometric mean of the major 2 diameters was recorded and the final number of PDMT was also checked.

Endocrine organ weights

At the end of the 2nd pregnancy, some mice in each group were killed by decapitation under the light ether anesthesia. Anterior pituitary, adrenals and ovaries were immediately removed and weighed.

Reproduction

Delivery interval, mother weight, litter size, still-birth rate, average pup weight at the 2nd parturition, and pup growth rate and rearing rate on days 12 and 20 of the 2nd lactation were recorded [14] in the experimental and the control mice of Experiment II.

Immunohistochemistry

The sections cut at 6 μm with cryostat microtome and fixed with 4% paraformaldehyde were incubated with 1% BSA/PBS for 20 min followed by the incubation with antibody to product of c-*jun* (Oncogene Sci., Manhasset, NY, USA: 3 μg/ml PBS), c-*fos* (Oncogene Sci., 3 μg/ml PBS) or N-*myc* (Cambridge Res. Biochem., Cambridge, UK: 10 μg/ml PBS) in a moisture chamber for 24 hr at 4°C. Horseradish peroxidase-labeled anti-rabbit IgG (Amersham, UK: 1:50) or anti-sheep IgG (Cappel, West Chester, PA, USA: 1:1000) was used for the second antibody to the antibody to the product of the respective oncogene. The reaction of peroxidase was carried out by diaminobenzidine for 5 min. The sections overlaid with normal rabbit serum and 1% BSA/PBS instead of the primary antibodies were set as the positive and the negative controls, respectively.

The degree of staining was graded as follows; (++) positive staining in almost cells of the tissue, (+) patchy positive staining of the cells and (−) no positive staining in all cells.

Statistics

All parameters were expressed in terms of mean ±SEM and the statistical significance of difference in the parameters between each experimental and the control groups was evaluated by the Student's t-test.

Simple correlation coefficients between some parameters were also calculated.

RESULTS

Growth of PDMT (Fig. 2)

No significant correlation was observed between the size of PDMT and the period of treatment in the experimental group, while in the control there was a positive correlation between parameters. The linear regression coefficient was also apparently higher in the control than in the experimental group.

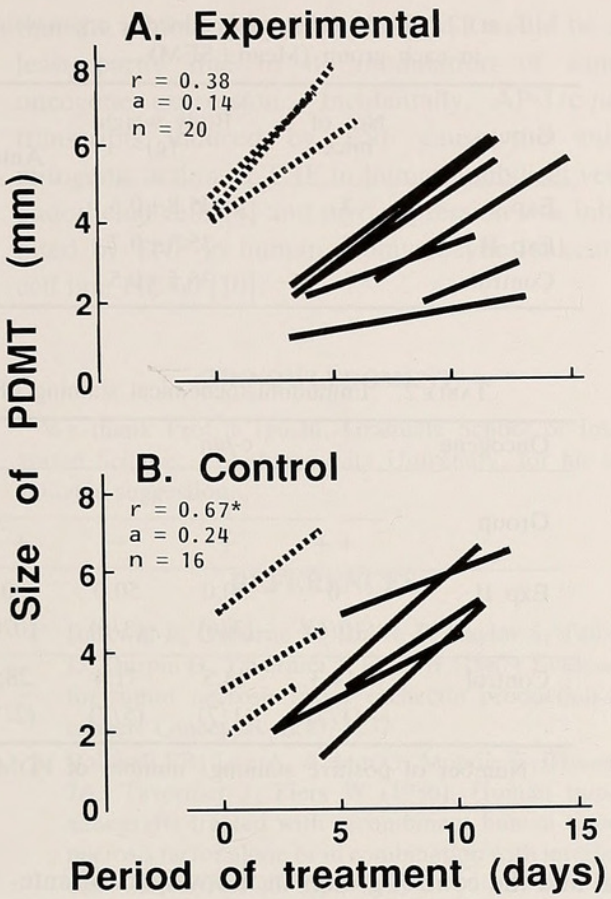


FIG. 2. Growth of PDMT in each group. ------: Experiment I, in which PDMT was already palpable at the start of TNF (vehicle) injection. ——: Experiment II, in which PDMT became palpable 2–9 days after the start of injection. r and a: Correlation and regression coefficients, respectively, between the size of PDMT and the period (days) of treatment. n: Number of estimates. *$P<0.05$.

The number of PDMT

The numbers of PDMT per mouse at the end of the 2nd pregnancy were 1.3±0.3 (3), 1.9±0.3 (7) and 2.4±0.3 (8) (mean±SEM and the number of mice in parentheses) in Experiments I, II and the control, respectively.

Endocrine organ weights (Table 1)

No difference was seen between the experimen-

TABLE 1. Body weight and endocrine organ weights of mother mice at the end of the 2nd pregnancy in each group (Mean±SEM)

Group	No. of mice	Body weight (g)	Endocrine organ weights (mg)		
			Anterior pituitary	Adrenals	Ovaries
Exp I	3	35.8±0.5	3.4±0.1	6.5±0.2	13.1±0.5
Exp II	4	35.5±0.7	3.4±0.1	5.7±0.3	12.9±1.0
Control	5	35.5±0.5	3.5±0.4	5.8±0.4	11.6±0.9

TABLE 2. Immunohistochemical staining of oncoproteins in PDMT in each group (%)

Oncogene	c-*jun*			c-*fos*			N-*myc*		
Group	Grade								
	++	+	−	++	+	−	++	+	−
Exp II	0 (0/6)[1]	50.0 (3/6)	50.0 (3/6)	0 (0/6)	0 (0/6)	100 (6/6)	0 (0/6)	0 (0/6)	100 (6/6)
Control	14.3 (1/7)	14.3 (1/7)	71.4 (5/7)	28.6 (2/7)	28.6 (2/7)	42.9 (3/7)	28.6 (2/7)	28.6 (2/7)	42.9 (3/7)

[1] Number of positive staining/ number of PDMT examined.

tal and the control groups in the weights of anterior pituitary, adrenals and ovaries at the end of the 2nd pregnancy.

Reproduction

Any reproductive parameter at the 2nd parturition and the pup growth rate and the rearing rate on day 12 or 20 of the 2nd lactation differed little between the experimental and the control groups of Experiment II (data not shown).

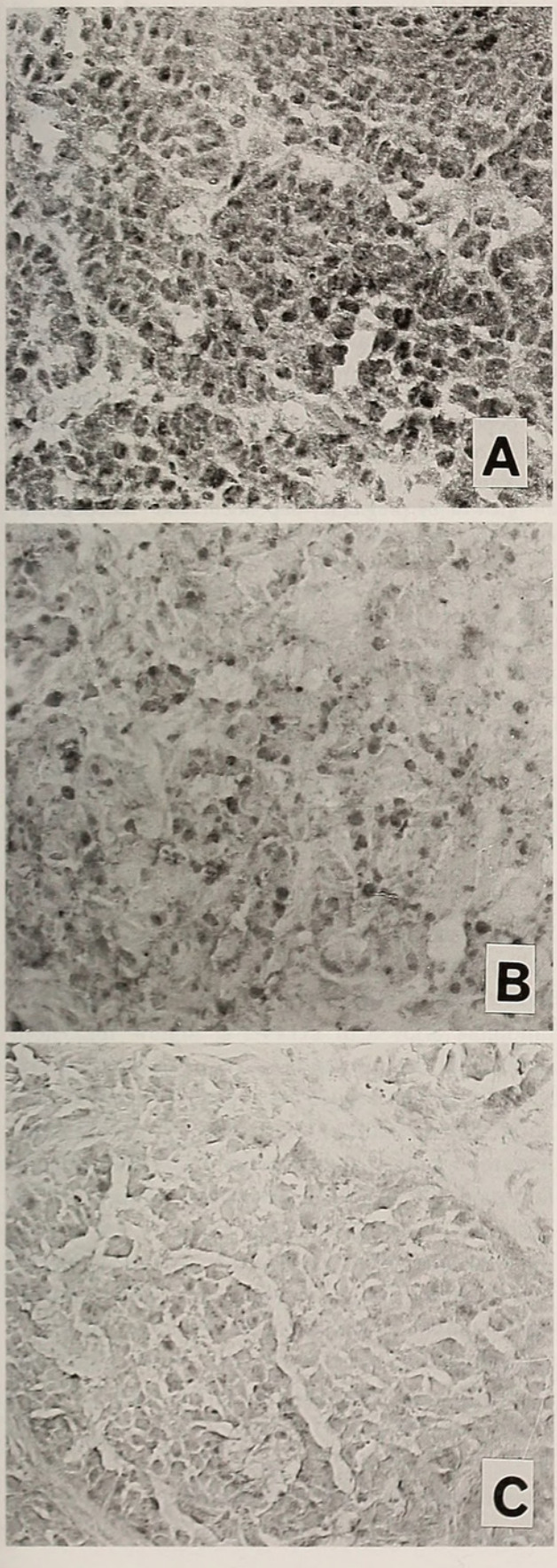
Immunohistochemistry of oncogene expression in PDMT (Table 2)

According to the standard shown in Fig. 3, the staining of c-*jun* product in PDMT of mice given TNF was more intense compared to the control. The positive staining of the product of neither c-*fos* nor N-*myc* was seen in PDMT of the experimental mice, while the positive staining of each oncogene product was 4 (57%) out of 7 in the control.

DISCUSSION

This study shows that the significantly positive correlation between the size of PDMT and the period of vehicle treatment observed in the control disappeared in the the experimental group treated with TNF. Furthermore, the linear regression coefficient was apparently lower in the latter than in the former. The number of PDMT also tended to be smaller in the experimental groups than in the control. All results indicate that TNF inhibits the growth of PDMT, of which effect is related to the period of treatment. Antitumor activity of TNF is different due to the injection period as well as the type of tumors [2, 11]. Recently, Goto *et al.* [7] found the dramatic changes of several proteins in PDMT associated with its growth and regression. TNF has been reported to be produced by some carcinomas including breast cancer [1]. Thus, it is plausible that PDMT may produce TNF, which, in turn, may participate in the regression of PDMT through the autocrine system.

The contribution of oncogenes to the development and growth of mamamry tumors has been suggested, while the mechanisms remain mostly to be solved [3, 6, 8, 15]. In this study, the immunohistochemical staining of products of both *fos* and *myc* was inhibited, while the staining of *jun* product was stimulated by TNF. The results suggest



that the inhibition by TNF of PDMT would be at least partly due to its modulation of some oncogene expression. Incidentally, AP-1/c-*jun* transcripts induced by TNF cause the antimitogenic action of TNF in human umbilical vein endothelial cells [4] and *myc* expression was inhibited by TNF in human promyelocytic leukemic cell line HL-60 [10].

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FIG. 3. Immunohistochemical staining of c-*jun* product in PDMT (×100).
A: Positive staining (++). Nuclei of almost cells are deeply stained.
B: Patchy positive staining (+). Nuclei of several cells are weakly stained.
C: Negative staining (−).

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