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THE ROLE OF APOPTOSIS IN THE INHIBITION OF A SECONDARY TUMOR BY CONCOMITANT RESISTANCE IN A MOUSE MODEL OF METASTASES

MARCELA FRANCO, PEDRO BEKINSCHTEIN, PEDRO D. di GIANNI, OSCAR D. BUSTUOABAD, SILVIA VANZULLI, RAUL A. RUGGIERO

Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires

Summary Resistance of tumor-bearing mice to a second tumor challenge, that is, concomitant resistance, was studied using the LB tumor model. In a secondary LB tumor implant inhibited by concomitant resistance an increase in the percentage of apoptotic cells and alterations in cell cycle distribution were observed. Similar alterations were observed in LB tumor cells incubated with serum from tumor-bearing mice. The data presented in this paper suggest that apoptosis is one of the mechanisms involved in tumor dormancy due to concomitant resistance.

Key words: apoptosis, concomitant resistance, metastases

Concomitant resistance is the phenomenon according to which a tumor-bearing host inhibits the growth of a secondary implant of the same tumor at a distant site[1]. The relevance of this model is that it can mimic the relationship between a primary tumor and its distant metastases[2, 3]. Concomitant resistance induced by weakly and non-immunogenic tumors has been described as non-specific and mediated by a mechanism presumibly unrelated to any known conventional immunological mechanisms[4, 5, 6, 7]. In previous papers[8, 9], we have demonstrated that BALB/c mice bearing a non-immunogenic lymphoma, called LB, generated a strong concomitant resistance. We have also demonstrated that serum from these LB-bearing mice had an inhibitory activity (not attributable to cytotoxic antibodies) on *in vitro* (^{3}H) thymidine incorporation by LB-tumor cells. This activity was proportional to the intensity of concomitant resistance and was mediated by factor/s of low molecular weight (1000-1200 D).

There is a growing body of evidence on the importance of apoptosis in tumor inhibition induced by chemotherapeutic drugs and radiation[10, 11] and by antiangiogenic factors[12].

The aim of this paper was to investigate whether apoptosis and cell cycle alterations were involved in the concomitant resistance induced by LB tumor. The role of apoptosis was evaluated a) *in vivo* in the secondary LB tumor implant and b) *in vitro* in LB cells incubated with serum from LB tumor-bearing BALB/c mice (LB serum).

BALB/c mice were s.c. inoculated with 10^6 LB tumor cells and were reinoculated with 10^5 LB cells in the opposite flank seven days later. The control group received this «second» implant only.

The percentage of cells with hypodiploid DNA content (apoptotic) in the secondary 7-day tumor was investigated using hypotonic Propidium Iodide (PI) fluorochrome staining and cytofluorometric technics as previously described by others[13, 14] and was confirmed by DNA electrophoresis and by fluorescence microscopy.

The secondary LB tumor, prevented to grow by concomitant resistance, showed a significantly higher percentage of apoptotic cells (18.1 ± 2.1) than that observed in the control group (3.9 ± 1.3) (mean ± SE). A representative histogram of DNA content is shown in Figure 1.

Similarly, a significant increase in the apoptotic population in LB tumor cells incubated with differ-

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Postal address: Lic. Marcela Franco, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, P. Melo 3081, 1425 Buenos Aires, Argentina

ent dilutions of LB serum during 7 hours was observed (Table 1) as compared with incubation in normal serum. Additional evidence of apoptotic cell death was obtained by gel electrophoresic analysis of the DNA degradation products, showing the typical «ladder» pattern and by fluorescence microscopy (data not shown).

A correlation between cell cycle alterations and apoptosis has been reported in various systems; in some, apoptosis occurs only after cell cycle arrest and in others, cells are preferentially susceptible to apoptosis at specific phases of the cell cycle[10, 11]. In this paper, we analyzed the cell cycle distribution of the non-apoptotic population of

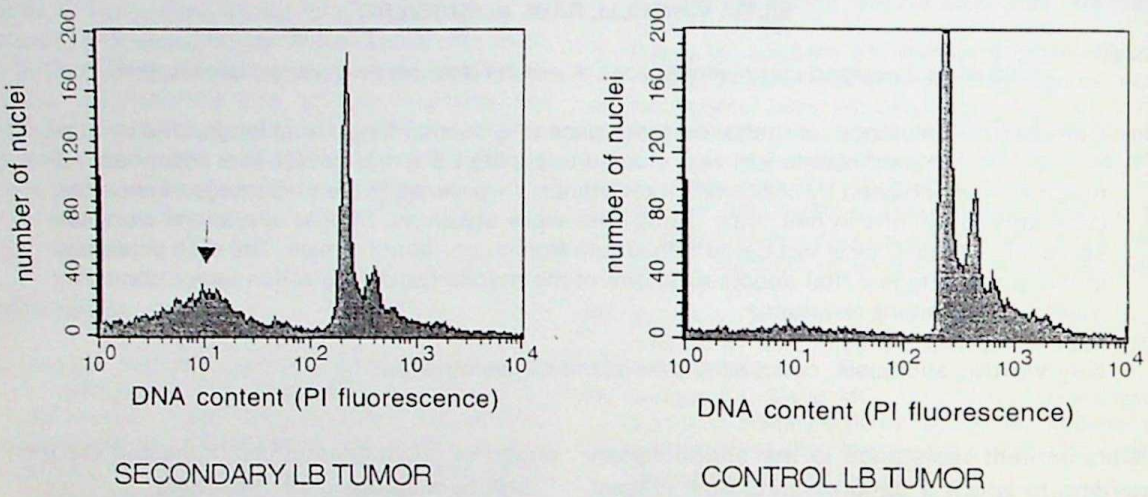

Fig. 1. Flow cytometric DNA fluorescence profiles of PI-stained LB tumor cells from a secondary LB tumor and its control in a representative experiment. Arrow indicates the population of nuclei with hypodiploid DNA content (apoptotic).

the secondary LB tumor and determined the existence of differences between experimental and control groups. In effect, there was a decrease in the percentage of cells in the G2-M phases and an increase in the proportion of cells in the S phase in the second tumor implant in mice bearing a primary LB tumor as compared with the control group (experimental group: G0-G1: 66.7 ± 1.4, S: 29.7 ± 0.6% ($P < 0.001$) and G2-M: 3.5 ± 1.3% ($P < 0.001$); control group: G0-G1: 75.6 ± 3.9%, S: 10.9 ± 4.9% and G2-M: 9.8 ± 1.1%) (Mean ± SE of 3 assays).

The non-apoptotic population of LB tumor cells incubated with LB serum also showed alterations in cell-cycle distribution: a significant decrease in the percentage of cells in G2-M compartment was observed (Table 1), in accordance with data obtained in *in vivo* experiments.

The lower proportion of cells in G2-M obtained both in *in vivo* and in *in vitro* assays are in accordance with previous data showing a decrease in the number of metaphases per field at the site of the secondary tumor implant[7].

Although the data presented here do not allow us to explain the alterations observed in cell cycle distribution, some considerations can be made. A special susceptibility to apoptosis of cells in G2-M (and likely, in G0-G1) could account for these results. On the other hand, we cannot discard that LB cells could go to apoptosis independently of the position in the cell cycle. In this case, the changes observed in the cell cycle could be the consequence of growth arrest that makes it difficult for LB cells to complete the S stage and pass through G2, raising the possibility that a «cytostatic» effect of LB serum could exist independently of its apoptotic effect.

Up to now, the identity of the factor/s responsible for the observed apoptosis needs to be established. The results reported herein suggest that

TABLE 1.— *Apoptosis and alterations in cell cycle distribution in LB-tumor cells incubated* in vitro *with different dilutions of LB serum or normal serum.*

serum	dilution	% apoptotic cells	% viable cells in each phase		
			G0-G1	S	G2-M
LB serum	1:2	30.6 ± 4.7*	62.5 ± 5.1	32.1 ± 5.8	5.3 ± 1.8*
	1:4	18.9 ± 3.5*	60.9 ± 6.0	34.2 ± 2.0	4.8 ± 1.1*
	1:8	14.7 ± 5.8	ND	ND	ND
normal serum	1:2	14.0 ± 2.5	62.9 ± 3.9	25.5 ± 3.2	11.7 ± 1.2
	1:4	11.0 ± 2.5	66.1 ± 3.6	22.7 ± 2.7	12.2 ± 1.4
	1:8	10.6 ± 3.2	ND	ND	ND

Valves were expressed as mean ± SE. LB tumor cells were incubated *in vitro* with different dilutions of LB serum or normal serum in a 7-hour assay. Incidence of apoptotic LB tumor cells treated with RPMI was 6.83 ± 1.4%. Percentages of LB tumor cells incubated with RPMI: G0G1: 71.5 ± 4.7; S: 18.5 ± 6.2; G2-M: 10.0 ± 2.8. (*: $P < 0.001$; Student t-test between LB serum and normal serum).

apoptosis is involved in the quiescence or dormancy observed in a secondary LB tumor inhibited by concomitant resistance and also suggest that serum factors from LB-tumor bearing mice —previously reported as associated with the phenomenon of concomitant resistance[8, 9] — could play a role in the observed apoptosis.

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Resumen

El rol de la apoptosis en la inhibición del tumor secundario por la resistencia concomitante en un modelo murino de metástasis

La resistencia concomitante es la capacidad de un individuo portador de un tumor, de inhibir el crecimiento de un segundo implante tumoral. En este trabajo se estudia el rol de la apoptosis en este fenómeno utilizando como modelo el tumor murino LB. Se observó, en el tumor secundario LB inhibido por resistencia concomitante, un aumento en el porcentaje de células apoptóticas y alteraciones en el ciclo celular. Se obtuvieron resultados similares cuando las células del tumor LB fueron incubadas con suero de ratones portadores del mismo tumor. Los resultados sugieren que la apoptosis está involucrada en el estado de tumor dormido característico del fenómeno de resistencia concomitante.

References

1. Gorelik E. Concomitant tumor immunity and the resistance to a second tumor challenge. *Adv Cancer Res* 1983; 39: 71-120.
2. Sugarbaker EV, Thornthwaite J, Ketcham AS. Cancer invasion and metastases. *In* Biological mechanisms and therapy. Day SB et al (eds), New York: Raven Press, 1977, pp 227-40.
3. Bonfil RD, Ruggiero RA, Bustuoabad OD, Meiss RP, Pasqualini CD. Role of concomitant resistance in the development of murine lung metastases. *Int J Cancer* 1988; 41: 415-22.
4. Prehn RT. Two competing influences that may explain concomitant resistance. *Cancer Res* 1993; 53: 3266-9.
5. Gorelik E. Resistance of tumor-bearing mice to a second tumor challenge. *Cancer Res* 1983; 43: 138-45.
6. Ruggiero RA, Bustuoabad OD, Bonfil RD, Meiss RP, Pasqualini CD. «Concomitant immunity» in murine tumours of non-detectable immunogenicity. *Br J Cancer* 1985; 51: 37-48.
7. O'Reilly MS, Holgren L, Shing Y, et al. Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994; 79: 315-28.
8. Ruggiero RA, Bustuoabad OD, Cramer P, Bonfil RD, Pasqualini CD. Correlation between seric antitumor activity and concomitant resistance in mice bearing non-immunogenic tumors. *Cancer*

Res 1990; 50: 7159-65.
9. Franco M, Bustuoabad OD, di Gianni PD, Goldman A, Pasqualini CD, Ruggiero RA. A serum-mediated mechanism for concomitant resistance shared by immunogenic and non-immunogenic tumors. *Br J Cancer* 1996; 73 (in press).
10. Gorczyca W, Gong J, Ardeltt B, Traganos F, Darzynkiowicz Z. The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res* 1993; 53: 3186-92.
11. Evans D, Dive C. Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating inmature thymocytes. *Cancer Res* 1993; 53: 2133-39.
12. O'Reilly M, Holmgren L, Chen C and Folkman J. Angiostatin induces and sustains tumor dormancy of human primary tumors in mice. *Nature Med* 1996; 2: 689-92.
13. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Meth* 1991: 139: 271-9.
14. Lindenboim L, Diamond R, Rothenberg E, Stein R. Apoptosis induced by serum deprivation of PC12 cells is not preceded by growth arrest and can occur at each phase of the cell cycle. *Cancer Res* 1995; 55: 1242-7.

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Sin la investigación pura, una Universidad o un país está condenado a la inferioridad. Prohibirla es una especie de suicidio nacional. Es obligar a importar los conocimientos y los técnicos, marchar a remolque, ser tributario, no tener independencia y jerarquía, faltar al deber de contribuir al adelanto de los conocimientos.

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El papel de la Ciencia. *Anales de la Sociedad Científica Argentina* 1950; CL: 197-209