META-TYROSINE. A POWERFUL ANTI-METASTATIC FACTOR WITH UNDETECTABLE TOXIC-SIDE EFFECTS

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Abstract Concomitant tumor resistance (CR) is a phenomenon in which a tumor-bearing host is resistant to the growth of secondary tumor implants and metastasis. While former studies have indicated that T-cell dependent processes mediate CR in hosts bearing immunogenic small tumors, the most universal manifestation of CR induced by immunogenic and non-immunogenic large tumors had been associated with an antitumor serum factor that remained an enigma for many years. In a recent paper, we identified that elusive factor(s) as an equi-molar mixture of meta-tyrosine and ortho-tyrosine, two isomers of tyrosine that are not present in normal proteins and that proved to be responsible for 90% and 10%, respectively, of the total serum anti-tumor activity. In this work, we have extended our previous findings demonstrating that a periodic intravenous administration of meta-tyrosine induced a dramatic reduction of lung and hepatic metastases generated in mice bearing two different metastatic murine tumors and decreased the rate of death from 100% up to 25% in tumor-excised mice that already exhibited established metastases at the time of surgery. These anti-metastatic effects were achieved even at very low concentrations and without displaying any detectable toxic-side effects, suggesting that the use of meta-tyrosine may help to develop new and less harmful means of managing malignant diseases, especially those aimed to control the growth of metastases that is the most serious problem in cancer pathology.

Key words: meta-tyrosine, concomitant tumor resistance, anti-metastatic effects

Resumen ***Meta-tirosina. Un poderoso agente anti-metastásico sin efectos tóxicos colaterales detectables.***
La resistencia concomitante antitumoral (RC) es el fenómeno según el cual un individuo portador de tumor inhibe el crecimiento de implantes tumorales secundarios y metástasis. Si bien desde hace tiempo se sabe que la RC inducida por tumores inmunogénicos de pequeño tamaño es generada por mecanismos inmunológicos dependientes de células T, por otro lado, la manifestación más universal de la RC, generada tanto por tumores inmunogénicos como no-inmunogénicos de gran tamaño, había sido asociada con un (unos) factor sérico antitumoral cuya naturaleza permaneció elusiva por años. En un trabajo reciente, nuestro grupo de trabajo identificó este factor como la mezcla equi-molar de meta-tirosina y orto-tirosina, dos isómeros de tirosina que no están presentes en proteínas normales y que demostraron ser responsables del 90% y 10%, respectivamente, de la actividad antitumoral total del suero. En este trabajo, continuamos nuestras investigaciones demostrando que la administración periódica de meta-tirosina reducía drásticamente el número de metástasis pulmonares y hepáticas en ratones portadores de dos tumores murinos altamente metastásicos y disminuía dramáticamente la mortandad (de 100% a 25%) de ratones con metástasis ya establecidas al momento de la extirpación quirúrgica del tumor. Estos efectos anti-metastásicos se lograron aun con muy bajas concentraciones de meta-tirosina y sin efectos tóxicos perceptibles, lo que sugiere que su uso puede ayudar a diseñar nuevas y menos nocivas estrategias para el tratamiento del cáncer, especialmente aquellas destinadas a controlar el crecimiento metastásico, que es el problema más grave en la enfermedad oncológica.

Palabras clave: meta-tirosina, resistencia concomitante antitumoral, efectos anti-metastásicos

Concomitant tumor resistance (CR) is the phenomenon according to which a tumor-bearing host inhibits or retards the growth of secondary tumor implants. It was first described by Ehrlich in 1906[1] but, apart from a few isolated papers[2, 3] this phenomenon remained virtually forgotten for about 60 years[4, 5]. After its renascence, some groups have studied this phenomenon demonstrating that both immunogenic and non-immunogenic tumors can induce CR in different animal models such as mice, rats and hamsters[6-8]. However, CR has not received much attention as compared with other areas of cancer research despite the fact that it has been detected in association with human

cancer and despite its relevance to the mechanisms of metastases control, taking into account that metastases could be considered as secondary tumor implants developed spontaneously during the primary tumor growth. In this sense, clinical and experimental evidence accumulated throughout the years has suggested that the removal of human and murine tumors may, upon certain circumstances, be followed by an abrupt increase in metastatic growth[9], suggesting that a primary tumor could exert a controlling action on its metastases. Metastatic growth is considered a far more serious problem than the original tumor because, for most cases, they ultimately prove to be fatal for the patient. In effect, prior to metastases, most cancers can be cured surgically and 5-year survival rates are about 90%. However, when a tumor has spread to different sites, those rates, even using some forms of systemic therapy such as chemotherapy, often fall below 15%[10]. Therefore, the understanding of the mechanisms underlying the phenomenon of CR could have a significant impact in the management of the malignant diseases.

Different hypothesis have been proposed to explain the phenomenon of CR. According to the immunological hypothesis, the growth of a tumor generates a specific antitumor immune response which even though it is not strong enough to inhibit the primary tumor growth, is still capable of preventing the development of a relatively small secondary tumor inoculum. The immunological hypothesis was originally proposed by Bashford in 1908[2] which, in turn, coined the term "concomitant immunity" by which this phenomenon has been known in the past. This interpretation is supported by solid evidence mainly based on experiments with strongly immunogenic murine tumors induced by chemical agents or viruses[8]. However, it does not provide a satisfactory explanation for the fact that CR has also been observed in association with spontaneous murine tumors of non-detectable immunogenicity[7, 11]. As for non-immunological explanations, basically two hypotheses have been formulated. Ehrlich[1] believed that nutrients essential for tumor growth are consumed by the primary tumor, making it difficult for a second implant to develop (atrepsis theory). Others[7, 12] have postulated that the primary tumor produce –or induce the production of– anti-proliferative nonspecific substances or anti-angiogenic molecules which limit the replication of tumor cells of the second inoculum. These non-immunological hypotheses can offer a putative explanation for the phenomenon of CR induced by non-immunogenic tumors but not for the specific inhibition of secondary tumor implants observed during the growth of immunogenic tumors.

For the last 30 years, we have studied, in our laboratory, the phenomenon of CR associated with the growth of 20 murine tumors displaying widely different degrees of immunogenicity, in an attempt to integrate the different hypotheses into a coherent picture. Our results[7, 8, 11] demonstrated that, two temporally separate peaks of CR are generated during primary tumor growth. The first peak was only induced by immunogenic tumors of small size ($\leq$ 500 mm³); it was tumor-specific and thymus-dependent and a typical immunological rejection was observed histologically at the site of the second tumor implant undergoing CR. On the other hand, the second peak of CR was induced by both immunogenic and non-immunogenic large tumors ($\geq$ 2000 mm³); it was tumor-non-specific and thymus-independent and the inhibition of the secondary tumor was not associated with an immunological cell infiltration, but with the presence of viable but non-infiltrating tumor cells (dormant tumor) located at the inoculation site between the skin and the muscular layer. Some years ago, an intermediate peak of CR was reported to be associated with a particular type of mild-sized tumors (1000-1500 mm³) that restrain secondary tumors indirectly, by limiting tumor neovascularization[12]. Although the mechanisms associated with the first and intermediate peaks of CR have been elucidated as T cell–dependent and angiostatin-dependent, respectively, the molecular basis of the most universal manifestation of CR, that is, the second peak, remained an enigma for many years. In former studies, we demonstrated that the second peak of CR correlated with the activity of a serum factor(s) that inhibited the *in vitro* and *in vivo* proliferation of tumor cells and that was unrelated to other well characterized growth-inhibitory molecules such as interferons, TNF-α, TGF-β, angiostatin, endostatin, etc., taking into account the larger molecular weight of the latter and other physical and biological properties[7, 8]. In a recently published work[13], starting from mice bearing a non-immunogenic lymphoma (called LB), that produces the strongest second peak of CR among all our tumor models, we identified such serum factor(s) as meta-tyrosine and ortho-tyrosine, two isomers of tyrosine that it is thought to be absent from normal proteins. Meta-tyrosine and ortho-tyrosine proved to be responsible for 90% and 10%, respectively, of the total serum anti-tumor activity, as demonstrated by *in vitro* and *in vivo* experiments on the growth of three different murine tumors that induce CR and on the growth of established spontaneous metastases generated by a fourth tumor that does not induce CR but is sensitive to the CR induced by other tumors.

In this work, in order to extend our previous observations concerning the anti-metastatic role of meta-tyrosine (herein we used only meta-tyrosine because its anti-tumor power proved to be 10 times more robust than that of ortho-tyrosine), we have carried out the following series of experiments:

a) Study of the dose-response of meta-tyrosine administration on the growth of spontaneous metastases generated by the presence of two highly metastatic murine tumors: the mammary carcinomas C7HI and LMM3, kindly provided by Dra. C. Lanari (*Instituto de Biología y Medicina Experimental*, Buenos Aires, Argentina) and

Dr. L. Colombo (*Instituto Ángel Roffo*, Buenos Aires, Argentina), respectively.

b) Evaluation of the effect of meta-tyrosine on the survival of LMM3-excised mice exhibiting established lung metastases at the time of surgery.

c) Analysis of putative cytotoxic side-effects associated with a periodic administration of meta-tyrosine.

a) BALB/c mice bearing a subcutaneous (s.c.) C7HI tumor for 40 days were divided in four groups that received, between days 40 and 60, a daily intravenous (i.v.) injection of three different doses of meta-tyrosine (3.3; 33 and 67 mg/kg) or saline (control). A fifth group was sacrificed at day 40 to evaluate the number of metastases at the onset of treatment. At day 60, all treated and control mice were sacrificed and macroscopic (diameter $\geq$ 0.1 mm, x 10) and microscopic (diameter < 0.1 mm, H&E x 100) lung metastases were counted. As shown in Table 1, all the doses of meta-tyrosine reduced drastically the number of macroscopic lung metastases as compared with controls. In fact, the higher dose *decreased* the number of metastases that had been observed at the onset of treatment (Table 1). Microscopic lung metastases (data not shown) as well as macroscopic hepatic metastases were also significantly reduced at the end of treatment; hepatic metastases (median [range]) in control: 8 [3-18] vs. treated (67 mg/kg/day): 0 (p < 0.001, Mann-WhitneyU-test). Similarly, BALB/c mice bearing a s.c. LMM3 tumor for 25 days were divided in three groups that received, between 25 and 45 days, a daily i.v. injection of meta-tyrosine (3.3 and 67 mg/kg) or saline. A fourth group was sacrificed at day 25 to evaluate the number of metastases at the onset of treatment. At day 45, all treated and control mice were sacrificed and metastases counted. As shown in Table 1 all the doses of meta-tyrosine reduced drastically the number of macroscopic lung metastases as compared with

controls (Table 1). Microscopic lung metastases (data not shown) as well as macroscopic hepatic metastases were also significantly reduced at the end of treatment; hepatic metastases in control: 10 [5-16] vs. treated (67 mg/kg/day): 0 (p < 0.001, Mann-Whitney U-test). It is worth to note that the administration of meta-tyrosine by the intra-peritoneal (i.p.) route was not effective to restrict metastatic growth.

b) Twenty five BALB/c mice were inoculated sc with 2 x 10^5 LMM3 tumor cells. Eighteen days later, 19 tumor-bearing mice were surgically operated to remove the tumor and the remaining 6 were sacrificed to evaluate the number of lung metastases at the time of surgery (mean [range] = 6 [3-10]). Then, the tumor-excised mice were divided into two groups. One group (n = 8) received, for the following consecutive 35 days, a daily i.v. injection of meta-tyrosine (67 mg/kg). The other group (n = 9) received saline. The surgical excision was very satisfactory since no tumor relapsed. As shown in Fig. 1, all controls died exhibiting high number of lung and hepatic metastases at 43 ± 8 days (mean ± standard error) after surgery. In contrast only two treated-mice died, while the other 6 mice remained alive without exhibiting signs of local or metastatic disease for, at least, eight months after surgery (p < 0.001 Log Rank test).

c) The highest dose of meta-tyrosine (67 mg/kg) used in our previous experiments was administered daily by the i.v. route for 42 days in BALB/c mice. At day 42, a sample of mice was sacrificed and the following organs - skin, liver, kidney, spleen, lung, bone marrow, small and large intestine – were investigated histopathologically. Neither histologic nor cytologic alterations were detected in any case, even when organs with high rate of renewal such as skin, bone marrow or small intestine were studied. Hematologic cell populations in blood and lymphoid populations in lymph nodes and spleen were not altered either,

TABLE 1.– *Effect of periodic i.v. administration of meta-tyrosine on the number of macroscopic lung metastases generated by C7HI and LMM3 tumor-bearing mice*

| Tumor | Control | N° of lung metastases (median [range]) | | |
| | | Meta-tyrosine (mg/kg/day) | | |
		3.3	33	67
C7HI	44.5 [17-119]	19.4 [5-34][a]	14.2 [5-29][b]	9.3 [2-26][c]
	(n = 40)	(n = 23)	(n = 32)	(n = 18)
LMM3	60.0 [44-100]	30.5 [9-64][a]	ND	17.3 [5-33][c]
	(n = 15)	(n = 10		(n = 7)

Number of macroscopic lung metastases at the onset of treatment: 17.3 [9-32] for C7HI (n = 17, day 40) and 25.0 [22-28] for LMM3 (n = 10, day 25).
n = number of mice.
ND: not done.
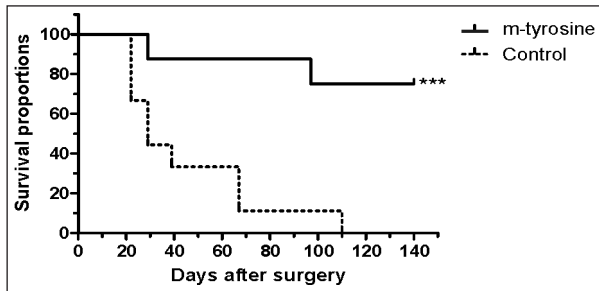*[a]: p < 0.05 vs. control, Mann Whitney U-test*
[b]: p < 0.01
[c]: p < 0.001

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LA TAPA

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Digitalis purpurea L.; *vulg.* dedalera, digital, cartucho, etc. Nativa de Europa, Asia y NO de Africa, introducida en América; crece invasora en el sur de Chile y las provincias vecinas de Neuquén y Río Negro en Argentina. Sus raíces, tallos, hojas, flores y semillas contienen glucósidos cardíacos que inhiben el transporte trans-celular de Na^+ y K^+, tienen efecto inotrópico positivo y regulan la actividad del sistema nervioso autónomo. Introducida por William Withering (1741-1799) del conocimiento popular a la medicina convencional:

"En el año 1775, se pidió mi opinión acerca de una receta familiar para curar la hidropesía. Me dijeron que era un secreto por mucho tiempo guardado por una anciana de Shropshire, quien algunas veces curó cuando los más convencionales médicos habían fallado. También fui informado que los efectos que produce eran violentos vómitos y purgantes, pero los efectos diuréticos parecían haber sido pasados por alto. La medicina estaba compuesta por veinte o más hierbas diferentes pero no fue difícil, para una persona familiarizada en estos temas, percibir que la hierba activa no era otra que la dedalera." *William Withering. An account of the foxglove, some of its Medical uses: with practical remarks on dropsy, and other diseases. Printed by M. Swinney, Birmingham, for G.G.J. and J. Robinson, London, 1785.* En: *www.botanicus.org/title/bl11202313*; 19-11-2014. Libre en línea.

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