Global Advanced Research Journal of Medicine and Medical Sciences (ISSN: 2315-5159) Vol. 6(12) pp. 341-351, December, 2017 Special Issue
Available online http://garj.org/garjmms

Full Length Research Paper

Revisiting the phenomenon of concomitant tumor resistance and its impact on established metastases of murine and human origin

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Accepted 8 December, 2017

Concomitant tumor resistance (CR) is a phenomenon in which a tumor-bearing host inhibits the growthof secondary tumor implants. The relevance of CR to mechanisms of metastases control has been highlighted by numerous observations showing that the removal of human and murine tumors may be followed by an abrupt increase in metastatic growth. This body of evidence suggests that, upon certain circumstances, a primary tumor would exert a controlling action on its metastases that can be considered as natural secondary tumor implants spontaneously developed during the primary tumor growth.In this article we revised both former and recent evidence accounting for this fact in both experimental and clinical settings and discussed the situations in which tumor removal would be or would not be recommended. In addition, we analyzed the different mechanisms historically proposed to explain CR especially focusing on the last investigations of our laboratory concerning the importance of tyrosine isomers as mediators of the phenomenon of CR and on their capacity to inhibit established metastases of both murine and human origin. Our investigations aimed to elucidate the molecular basis of the phenomenon of CR might stimulatethe design of new strategies aimed to limit the development of metastases,an issue of critical importance for patients afflicted by malignant diseases.

Keywords: Metastases, concomitant tumor resistance, murine and human tumors, tyrosine isomers.

THE PHENOMENON OF CONCOMITANT RESISTANCE

HISTORICAL BACKGROUND

The phenomenon according to which a tumor-bearing host inhibits or retards the growth of secondary tumor implants is called concomitant tumor resistance (CR) (Prehn, 1993; Chiarella et al., 2012). CR was originally described by Paul Ehrlich at the turn of the 20th century (Ehrlich, 1906) but, apart from a few isolated reports (Bashford et al., 1908 ; Woglom, 1929) it remained

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virtually forgotten for about 60 years until it was re-discovered in the 60'sby Southam (1964), Gershon (1967) and others (Brunschwig et al., 1965; Lausch and Rappe, 1969).

Since that moment on, 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 (Gorelk, 1983; Keller, 1985; Ruggiero et al., 1985; Franco et al., 1996). However, even after its renascence, CR has not received much attention as compared with other areas of cancer research despite its putative 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 (Gorelik, 1982; Bonfil et al., 1988; Di Gianni et al., 1999; Kaya et al., 2004; Demichelis et al, 2005; Peeters et al., 2008). In this regard, as shown in some detail in the following sections, experimental and clinicalevidence accumulated throughout the years has suggested that the removal of animal and human tumors may, uponcertain circumstances, be followed by an abrupt increase in metastatic growth (Ketcham et al., 1961; Sugarbaker et al., 1977; Lange et al., 1980; Coffey et al., 2003; Demicheli et al., 2008; Retsky et al., 2008), suggesting that a primary tumor could exert a controlling action on its metastases.

CONCOMITANT RESISTANCE AGAINST METASTASES

EXPERIMENTAL EVIDENCE

Accelerated growth of metastases following excision of the primary tumor was described more than a century ago by Tyzzer (1913). He observed that, although the surgical removal of a primary tumor prolonged the survival of mice, the size of the individual metastatic nodules was larger than in tumor-bearing mice. Similar results were obtained a decade later by Tadenumaand Okonogi(1924) also working with murine models.In the last 50-60 years, these pioneer experiments were confirmed in many tumor models in which detectable metastases were either present or inapparent (occult metastases) at the time of tumor removal (Ketcham et al, 1961; Gorelik, 1982; Gorelik, 1983; Bonfil et al, 1988; Di Gianni et al, 1999).Most experiments were carried out using subcutaneous (s.c.) primary tumors but there were some studies in which intra-peritoneal primary tumors were utilized.

A) *Detectablemetastases are present in tumor-bearing animals and metastatic growth is enhanced upon primary tumor removal*

This possibility is associated with tumors that were similar to those studied originally by Tyzzer and Tadenuma and Okonogi. Theirbiological behavior has been reviewed previously (Gorelik, 1982; Gorelik, 1983, Ruggiero et al., 2012; Chiarella et al., 2012) and can be summarized as follows. When s.c.tumors were removed before the establishment of metastases, the surgery was curative. On the other hand, when metastases have already spread and settled in different tissues and organs, surgery failed to cure the animals and the outcome of that procedure was dependent on the size of the local tumor at the time of removal. When small tumors were concerned, the lungs and other organs were left with very few metastatic cells as compared with those in tumor-bearing animals in which the primary tumor continued to shed numerous cells into the circulation. In consequence, the total mass of proliferating metastatic cells in tumor-bearing animals exceeded the growth of the fewer cells existing in tumor-excised animals. At this stage, tumor excision significantly prolonged the survival of mice.When medium-sized tumors were removed,equilibrium could be reached between the effect of suppression exerted by the primary tumor and the shedding of new potentially metastatic cells. In consequence, the total mass of proliferating metastatic cells was similar in both tumor-bearing and tumor-excised animals because although tumor-excised animals displayed fewer metastatic foci, each focus was of larger size. At this stage, tumor removal still, although modestly, prolonged the survival of the operated animals, presumably because even though both metastatic masses were similar, the presence of the primary growing tumor was deleterious for the health of the host. Finally, when large tumors were removed, a higher number and size of visible metastatic nodules than those present in tumor-bearing animals, were observed. This reflects the fact that at that stage tumor excision would promote the growth of visible metastatic foci as it would also induce non-visible ones to be large enough to be countable. In contrast, the presence of the primary tumor would limit the growth of visible metastases and would prevent the emergence of new visible ones from very small undetectable ones. At this stage, tumor excision resulted in a significantly reduced survival of the operated animals.

B) *No detectable metastases are present in tumor-bearing animals but metastatic growth is induced to be detectable upon primary tumor removal*

Some tumor models display this behavior (Gorelik, 1982; Gorelik, 1983). In our experience, mice bearing some methycholanthrene-induced fibrosarcomas do not display detectable metastatic foci neither in lung nor elsewhere. However, after surgical tumor extirpation, metastases begin to appear, especially in lung. Similarly, some years

ago, Keller (1985) demonstrated, using a rat DMBA-induced fibrosarcoma, that no detectable metastases beyond regional inguinal lymph nodes could be found in tumor-bearing rats, whereas on the contrary, in tumor-excised rats, metastases spread into various anatomical distant organs and tissues including contra-lateral lymph nodes.

CLINICAL EVIDENCE

In clinical settings it is not easy to evaluate the impact of tumor removal on the kinetics of metastatic growth because surgery is one of the primary treatment modalities for solid tumors. In consequence, studies comparing metastatic growth in patients with non-excised tumors (expectant management) with those after tumor resection (surgical management) are very infrequent, although some of them are available in the literature. As a whole, these studies together with indirect evidence accumulated for the last 40 years, suggest that the two possibilities mentioned in the precedent section (see Experimental Evidence) may also be associated with human cancer.

For example, Iversen et al (1995) found no benefit with radical prostatectomy over expectant management, for adenocarcinoma of the prostate in a follow-up study which followed 111 patients for 23 years. Similarly, Demicheli et al (2008) and Retsky et al (2008) examined the death-specific hazard rates in patients with breast cancer that had underwent mastectomy alone with those of non-operated patients obtained from an accepted historical database. The non-operated patients (expectant management) exhibited a single peak between the fourth and the fifth year in the hazard rate for death. In contrast,a two peak hazard was detected in the operated patients: the first occurred between the third and the fourth year after surgery followed by a second peak at the eighth year. These experiments suggest that the natural history of breast cancer could, in some way, be adversely affected by the primary tumor removal. A recent debate concerning the utility of primary tumor removal in patients with breast cancer that present with distant metastases (stage IV) at diagnosis, has highlighted the problem of CR in human cancer (Nguyen and Truong, 2011). An obvious advantage of surgical treatment is the reduction of levels of circulating tumor cells released by the tumor, which can be seeded as metastatic foci. In addition, surgical resection can reduce different symptoms including pain, ulceration and lymphoedema that may adversely impact quality of life and function and can also reduce potential immunosuppressive factors released by the primary tumor that may affect putative anti-tumor immune responses. On the other hand, a putative disadvantage of surgery is based on the fact that it can promote the progression of metastases. In effect, surgery upregulates adhesion molecules in target organs, recruits immune cells capable of entrapping tumor cells and induces changes in cancer cells themselves to enhance migration and invasion to establish at the target site. In addition, surgical trauma induces local and systemic inflammatory responses that can also contribute to the accelerated growth of residual and micrometastatic disease (Tohme et al., 2017).

Up to date, the clinical studies aimed to solve this controversy showed that tumor removal may improve the survival in patients with breast cancer with stage IV but only in those displaying small primary tumors and limited metastatic load. When larger primary tumors and more metastatic load are concerned, surgery is not recommended (Nguyen and Truong, 2011).

Similar observations have been made concerning colorectal carcinomas. In effect, reported data from the literature support the view that primary tumor resection (PTR) in colorectal cancer with synchronous unresectable metastases should be discussed and validated by a phase III trial in selected patients exhibiting asymptomatic primary tumor, age ≤ 70 years, World Health Organization performance status (WHO-PS)< 2, no extra-hepatic metastatic disease and liver burden of less than 50%. In these patients, PTR, when performed laparoscopically and after preoperative immuno-nutrition, may lead to an increased overall survival. In all other cases, reported postoperative mortality and morbidity rates related to PTR are high and up-front chemotherapy with the primary tumor left in place may represent the more reasonable option (Mestier et al., 2014).In another study, a total of 116 patients with synchronous colorectal liver metastases were identified of which 49 received an upfront primary tumor resection and 67 received neo-adjuvant chemotherapy. The conclusion of this study indicated that tumor resection resulted in progressive disease (Slesser et al., 2014) suggesting that metastatic growth was enhanced after tumor removal.

In many other cancers, it has not possible to evaluate directly the kinetics of metastatic growth after primary tumor removal because of the lack of control non-operated patients. However,incidental but suggestive evidence has been reported.For example, Sugarbaker et al (1977) reported a clinical case of a 26 year-old male with a melanoma in the scalp. The disease was clinically localized and evaluation revealed no disseminated metastases. A wide excision and graft was performed; six weeks after the operation, numerous subcutaneous nodules as well as visceral metastases appeared and the patient died shortly after. In the same way, partial spontaneous regression of a primary melanoma is actually considered a bad prognosis sign (Chiarella et al., 2012). Lange et al (1980) reported a study of eight patients who underwent cytoreductive surgery for testicular cancer; in each case, the surgical procedure led to a very faster growth of regional and distant residual disease than that expected by assuming an uninterrupted natural growth of these residual tumors. Similar findings

in patients with epithelial ovarian cancer (Hoskins, 1989) led to some investigators to urge caution with respect to cytoreductive surgery. The above clinical studies together with similar investigations carried out with patients affected by similar or other malignancies strongly suggest that acceleration of metastatic growth may be the undesired outcome of surgical removal of many common human cancers such as melanomas, osteosarcomas and breast, testicular, ovarian, lung, colorectal and bladder carcinomas (Chiarella et al., 2012; Ruggiero et al., 2012).

As a whole, all the above mentioned experimental and clinical results are in agreement with the fact that a primary tumor usually exerts a phenomenon of CR against its own metastases. However, the removal of a primary tumor growing in animals or patients with detectable or occult metastases can increase or decrease the survival of both tumor-bearing animals and patients depending on the primary tumor mass and the number of metastatic foci present at the time of surgery. When the tumor is small and the metastatic load is relatively low, surgery is recommended but when the tumor is large and the metastatic load is high, surgery might not be recommended because the deleterious effects (acceleration of metastases) produced by the withdrawing of the tumor usually overcomes the putative beneficial effects of tumor removal.

Although the phenomenon of CR against the growth of metastases has been observed in many experimental systems and it has also been suspected in many clinical situations, on the other hand, there are also some experimental and clinical evidence that the presence of a tumor may not exert any effect or even a stimulating effect (Concomitant enhancement, CE) on their metastases (Ando et al., 1979; Janik et al., 1981; McAllister et al., 2008; Elkabets et al., 2011). In such cases, tumor removal would not induce any acceleration or even it would induce an inhibition of metastatic growth. In clinical settings, few putative examples of CE have been reported. Most of them have been related to occasional suspected regressions of hepatic and/or pulmonary metastases following nephroctomy for renal cell carcinoma (Lekanidi et al., 1997; Wyczolkowski et al., 2001; Lekanidi et al., 2007; Ray and Gosh, 2014). In our laboratory, we have demonstrated the presence of both CR and CE phenomena in some tumor-bearing mice, depending on the ratio between the mass of the larger primary tumor mass relative to that of the smaller secondary one, with high ratios rendering inhibition and low ratios inducing stimulation of the secondary tumor. However, in our experience, the magnitude of this stimulatory effect, whenever it is present, proved to be rather modest as compared with the magnitude of the inhibitory effect (Bruzzo et al., 2010). In consequence, taken together, the available experimental and clinical evidence suggest to us that CR would be more likely than CE to govern the behavior of animal as well as commonly occurring human tumors.

MECHANISMS ASSOCIATED WITH THE PHENOMENON OF CR

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 (for example, chemotherapy or immunotherapy), often fall below 15% (Chen et al., 2011). Taking into account that the behavior of tumor cells re-inoculated as secondary tumor implants into animals bearing a primary tumor mimics the situation observed during metastases formation, it appears that an understanding of the mechanisms underlying the phenomenon of CR could provide insight into the mechanisms that could inhibit the growth of metastatic cells in the presence of a primary tumor. This knowledge in turn could have a significant impact in the design of new strategies aimedto limit the development of metastases and, in consequence, to improve 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. This explanation is not very different from that of conventional immunological rejection of allogeneic tumors in naive mice or immunogenic syngeneic tumors in pre-immunized animals. The immunological hypothesis was originally proposed by Bashford in 1908 (Bashford et al., 2008) 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 (Gorelik, 1983; Franco et al., 1996). 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 (Keller, 1985; Ruggiero et al., 1985; Franco et al., 1996; Ruggiero et al., 1996).

Non-immunological explanations rely mainly on two hypotheses.

Ehrlich [1906] and Tyzzer [1913] believed that nutrients essential for tumor growth are consumed by the primary tumor, making it difficult or impossible for a second implant to develop (atrepsis theory). The term *“atrepsis”* was coined by Joseph Parrot in 1874 to describe malnutrition, especially in infants. A support for the

atrepsis theory to explain CR is associated with the fact that a progressive tumor is a trap for glucose, nitrogen and other nutrients. In this way, all attempts to correct the weight loss in tumor-bearing organisms by supplying different nutrients by the i.v. route resulted in acceleration of tumor growth (Gorelik, 1983). Taking into account that there is convincing evidence that nutrients restriction may be accompanied by inhibition of tumor growth, it is possible that in the setting of a severe systemic biochemical disturbance generated by the primary tumor, the condition for the proliferation of re-inoculated tumor cells (secondary tumor implants) cannot be as favorable as in control animals.

Others (DeWys, 1972; Gorelik, 1983; Ruggiero et al, 1990; O'Reilly et al., 1994; Ruggiero et al., 2011) have postulated that the primary tumor produces – or induces the production of – anti-proliferative nonspecific substances or anti-angiogenic molecules which limit the replication of tumor cells of the second inoculum. The idea that a tumor induces systemic effects by the production of some kind of substances was originally suggested by Nakamura and Fukuoka in the 50' in their concept of cancer toxohormone whose circulating concentration should rise with increased tumor mass (Nakahara and Fukuoka, 1958). Some years later, DeWys (1972) suggested that some of those substances could influence tumor growth rate. In his study, carried out in mice and using a highly metastatic lung carcinoma, he observed a slowing of the growth rate of both spontaneous metastases and artificial secondary tumor implants in the presence of a primary tumor.The slowing of metastatic growth was proportional to the primary tumor mass and it was observed even though some of these metastatic foci were microscopic in size. Host immunological factors did not seem to be involved, since growth of this tumor could not be prevented by specific pre-immunization. Following removal of the primary tumor, the slowing of tumor growth was reversible in both the spontaneous and the simulated metastases, suggesting that the primary tumor released non-immunological systemic factors into the circulation that limited the growth of natural (metastases) and artificial secondary tumor implants. More recently, the concept of a substance associated with the phenomenon of CR was re-inforced by the work of Folkman's group that demonstrated that the murine Lewis carcinoma could inhibit the growth of its metastases by restraining the neo-vascularization of the metastases through the action of a circulating 38 kD protein called angiostatin (O'Reilly et al, 1994) that is produced by cleavage of a larger protein, plasmin, itself a fragment of an even larger protein called plasminogen.

Taken together,these non-immunological hypotheses can offer a putative explanation for the 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 many murine and human tumors (the latter growing into immune-deficient mice) in an attempt to integrate the different hypotheses into a coherent picture. Our results, summarized in Table 1 and reported, at least in part, in former papers (Meiss et al., 1986; Franco et al., 1996; Ruggiero et al., 1990; Franco et al., 2000; Gueron et al., 2017) demonstrated that, two main temporally separate peaks of CR are generated during primary tumor growth. The first peak was only induced byimmunogenic tumors of small size (≤ 500 mm^3); it was tumor-specific and thymus-dependent – as it was exhibited in euthymic but not in nude mice - , its intensity was proportional to tumor immunogenicity and a typical immunological rejection - associated with extensive necrosis and a profuse infiltration with polymorphonuclear granulocytes and mononuclear cells -was observed histologically at the site of the second tumor implant undergoing CR. Furthermore, the kinetics of appearance and disappearance of the first peak of CR paralleled the kinetics of appearance and disappearance of specific cytotoxic antibodies and cell-mediated cytotoxicity against the tumors.

On the other hand, the second peak of CR was induced by both immunogenic and non-immunogenic large tumors (≥ 2000 mm^3); it was tumor-non-specific and thymus-independent – as it was exhibited in both euthymic and nude mice -,it did not correlate with tumor immunogenicity and its intensity was proportional to the primary tumor mass: the larger the primary tumor, the stronger the inhibition of the secondary tumor. Further, the inhibition of the secondary tumor by a large primary tumor was neither associated with a massive or focal necrosis nor with any host cell infiltration, contrasting with a classical immunological rejection. Instead, the secondary tumor implant remained in a dormant-like state, with viable but non-infiltratingtumor cellsplaced at the inoculation site between the skin and the muscular layer.Occasionally some apoptotic tumor cells began to appear after 24h of inhibition.

Some years ago, an intermediate peak of CR was reported to be associated with a particular type of mid-sized tumors (1,000-1,500 mm^3) – typically the Lewis lung carcinoma - that restrain secondary tumors indirectly, by limiting tumor neovascularization (O'Reilly et al, 1994). 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 intensity of the second peak of CR correlated with the activity of a serum factor (or factors), different from antibodies, complement or other well characterized growth inhibitory molecules, that inhibited the *in vitro* and *in vivo*

Table 1. Origin, level of immunogenicity and intensity of concomitant tumor resistance induced by 20 murine tumors of different histological type and three human tumor lines growing in nude mice.

Tumor	Origin	Immunogenicity	Concomitant tumor resistance	
			1º Peak	2ª Peak
Murine				
L15-A[1]	Allogeneic	Very strong	Very high	Very high
MC-D[2]	Induced by MC[a]	Very strong	Very high	Moderate
MC-C[2]	Induced by MC[a]	Very strong	High	High
MNU-MPA[3]	Induced by MNU-MPA[b]	Moderate	Moderate	Moderate
MC-B[2]	Induced by MC[a]	Moderate	Moderate	Moderate
S-180-O[2]	Spontaneous	Moderate	Moderate	Moderate
MNU[3]	Induced by MNU[c]	Weak	Low	Moderate
M3[3]	Spontaneous	Weak	Low	Moderate
LMM3[3]	Spontaneous	Weak	Low	Absent
CS[3]	Induced by MMTV[d]	Weak	Low	High
C7HI[3]	Induced by MPA[e]	Undetectable	Absent	Absent
PX[2]	Induced by GC[f]	Undetectable	Absent	Moderate
S-180-N[2]	Spontaneous	Undetectable	Absent	Moderate
P388[1]	Induced by MC[a]	Undetectable	Absent	Very high
CM[3]	Spontaneous	Undetectable	Absent	High
CEP[3]	Spontaneous	Undetectable	Absent	High
CEI[3]	Spontaneous	Undetectable	Absent	High
CPV[3]	Spontaneous	Undetectable	Absent	Moderate
L15-S[1]	Spontaneous	Undetectable	Absent	High
LB[1]	Spontaneous	Undetectable	Absent	Very high
Human				
KB[α]	Spontaneous	----------	Absent	High
Calu-6[β]	Spontaneous	----------	Absent	High
PC3[γ]	Spontaneous	----------	Absent	Very high

1. Lymphoma-leukemia
2. Fibrosarcoma
3. Carcinoma
a. MC = Methylcholanthrene
b. MNU-MPA = N-methyl-N-nitrosurea + medroxyprogesterone acetate
c. MNU = N-methyl-N-nitrosurea
d. MMTV = Murine mammary tumor virus
e. MPA = medroxyprogesterone acetate
f. GC = glass cylinder
α. Nasopharingeal carcinoma
β. Lung carcinoma
γ. Prostatic carcinoma
More details of the tumors are given elsewhere: see quotations Ruggiero et.al (1985, 1990, 2011, 2012), Meiss et al. (1986), Bonfil et al. (1988), Franco et al. (1996, 2000), DiGianni et al. (1999), Chiarella et al. (2012), Gueron et al. (2017).

proliferation of tumor cells. Further, mice bearing tumors that produced CR and such inhibitory serum factors, could display detectable or undetectable metastases but metastatic growth was strongly enhanced after tumor removal. Reciprocally, when the serum inhibitory activity was absent – the only two cases were mice bearing two highly metastatic tumors–the second peak of CR did not appear (Bonfil et al., 1988; Franco et al., 1996; Di Gianni et al, 1999). Further, after surgical extirpation of these tumors, growth of metastases was not stimulated.

These results suggested a direct correlation among the second peak of CR, the capacity to restrain the growth of metastases and the titer of serum growth inhibitory activity. Very interestingly, metastases produced by the two tumors that did not produce CR, were significantly inhibited by both the concomitant presence of unrelated tumors that induced CR and by the daily administration of serum from mice bearing these unrelated tumors, which displayed a high titer of growth inhibitory activity.

In recently published works [Ruggiero et al., 2011;

Gueron et al., 2017), we identified the anti-tumor serum factors associated with CR as a rather equi-molar mixture of *meta-tyrosine* (*m-tyr*) and *ortho-tyrosine* (*o-tyr*), two unnatural isomers of tyrosine (*p-tyr*), unnatural meaning that it is thought that they are absent from normal proteins (Gurer-Orhan et al., 2006; Bertin et al., 2007). We carried out this characterization starting from mice bearing a non-immunogenic murine lymphoma that produces the strongest second peak of CR among all our murine tumor models (Ruggiero et al., 2011) and also from nude mice bearing a human prostatic carcinoma that produces the strongest CR among all the human lines tested (Gueron et al., 2017). We could demonstrate that in both cases *m-tyr* and *o-tyr* were responsible for 90% and 10%, of the total antitumor activity of the serum, respectively, as determined by the inhibition of both the*in vitro* proliferation of many different murine and human tumor cells and the *in vivo* growth of subcutaneous tumor implants.

THERAPEUTIC EFFECT OF *M-TYR* AND *O-TYR* ON ESTABLISHED METASTASES OF MURINE AND HUMAN ORIGIN

The first evidence of the therapeutic value of *m-tyr* and *o-tyr* on established metastases was obtained using mice bearing different murine metastatic tumors growing subcutaneously. These tumors did not produce CR against experimental secondary tumor implants but they were very sensitive to the CR induced by unrelated tumors. When a periodic treatment of these tumor-bearing mice with *m-tyr* or *o-tyr* was initiated at the time when metastatic foci were already present in both lung and liver, a striking inhibition of metastatic growth was observed three and four weeks after the onset of the treatment (Machuca et al., 2015; Ruggiero et al., 2015; Gueron et al., 2017).

Similar results were observed on human metastatic cells (Gueron et al., 2017; Strazza et al., 2017). In one series of experiments, the inhibitory effect was achieved against experimental metastases produced by the intravenous inoculation of human prostatic tumor cells taking into account that this human tumor line does not produce metastases spontaneously neither in nude nor in SCID Nod gamma mice. In another series of experiment, using a human breast carcinoma that produces metastases spontaneously in SCID Nod gamma mice, periodic treatment with *m-tyr* resulted in a significant inhibition of lung metastases 3 weeks after the onset of the treatment.

The most impressive evidence of the therapeutic value of *m-tyr* and *o-tyr* was obtained in an experiment aimed to mimic a putative clinical situation. Part of this experiment – the data concerning the effect of *m-tyr* - was presented as *preliminary report* or *brief communication* in the Medicina (Bs.As.) journal (Machuca et al., 2015). The part corresponding to *o-tyr* has not ever been previously published. Taking into account that, according to the regulations of Medicina (Bs.As.) journal, data presented as *brief or preliminary communications* can be re-published in a full article in another journal, we presented in Figure 1, as illustrative information, the data corresponding to the effect of the periodic inoculation of both *m-tyr* and *o-tyr* on the survival of mice that had been operated from a metastasizing mammary murine carcinoma –at the time when metastases had already settled in lung and liver.

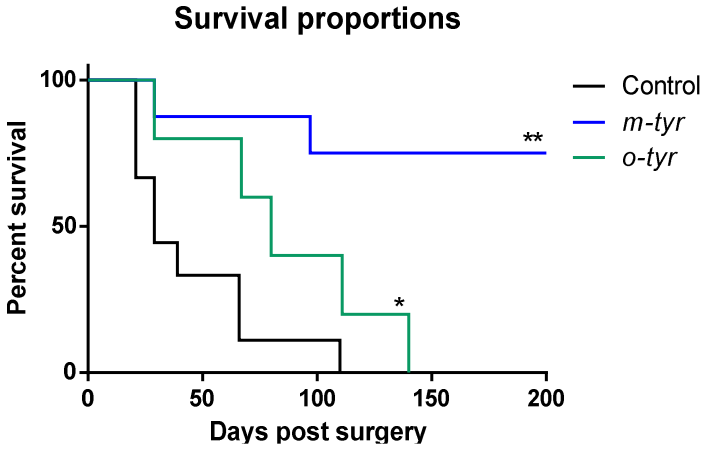

Figure 1. Percent survival of mice after surgical excision of LMM3 tumors.

The experiment can be summarized as follows: Twenty eight (28) mice were inoculated s.c. with $2x10^5$ LMM3 tumor cells(LMM3 is a highly metastatic murine mammary adenocarcinoma of spontaneous origin, see Machuca et al 2015; Gueron et al, 2017). Eighteen days later 22 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 three groups. One group (n = 8) received, for the following consecutive 35 days, a daily i.v. injection of *m-tyr* (67 mg/kg). The second group (n = 5) received, for the following consecutive 35 days, a daily i.v. injection of *o-tyr* (67 mg/kg). The third group (n = 9) received saline (control). The figure shows the percentage of the survivors of m-tyr-treated, o-tyr-treated and control mice (ordinate) as a function of the days after surgery (abscissa). The surgical excision was very satisfactory since no tumor relapsed. Death of mice was associated with lung and hepatic metastases. *:Difference between o-tyr-treated and control was $p<0.02$; **Difference between m-tyr-treated and control was $p<0.002$; Log Rank test.

As shown in Figure 1, all controls died rapidly after surgery with a median [range] of 29 days exhibiting high number of lung and hepatic metastases. In contrast, only two *m-tyr* treated-mice died (at days 29 and 97 after surgery), while the other 6 mice remained alive without exhibiting signs of local or metastatic disease for the rest of their lives. ($p<0.002$ vs. Control; Log Rank test). In effect, when these mice were sacrificed at 22 months old (that is about 18 months after the end of the treatment) no metastatic foci were detected neither in lung nor in liver nor elsewhere. As for *o-tyr* treated mice, all mice died although later than controls, with a median [range] of 80 days [29-140] ($p<0.02$; Log Rank test).

When treatment with m-tyr was initiated later, when the metastatic load were three times larger than in the first experiment, a significant anti-metastatic effect was also achieved and a significant percentage of treated mice (50%) survived at least six months after all controls had died (Gueron et al., 2017).

The therapeutic value of these and similar experiments is stressed by the fact that the striking antitumor effects mediated by *m-tyr* and *o-tyr* were observed without exhibiting any detectable toxic-side effects. In effect, the highest dose of *m-tyr* and *o-tyr* (67 mg/kg) - that we had used in our previous experiments aimed to control metastatic growth - 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 as well as different physiological variables in bloodwere not altered either, as evaluated by clinical analysis, direct microscopic observation and/or flow cytometry. In the same way, *m-tyr*-treated and *o-tyr*-treated mice did not display a lower humoral (titer of antibodies against sheep red blood cells) or cellular (delayed hypersensitivity) immune response than untreated controls (Machuca et al., 2015;Gueron et al., 2017; Strazza et al., 2017).

MECHANISMS OF TUMOR INHIBITION ASSOCIATED WITH TYROSINE ISOMERS

The inhibition exerted by *m-tyr* and *o-tyr* on tumor growth mimics the inhibition produced by CR. In both cases, tumor inhibition was primarily associated with the presence of a high proportion of tumor cells in G0, a decrease in G2-M phases, and an increase or accumulation of cells in the S-phase, considered the consequence of an S phase arrest (Ruggiero et al, 2011).

A molecularanalysis (Ruggiero et al., 2011; Gueron et al., 2017) showed that the antitumor effects mediated by*m-tyr* and *o-tyr*on murine and human tumor cells were mediated, at least in part, by an early inactivation of p-STAT3and down regulation of theNFκB//NOTCH axis that are constitutively activated in many tumor cells. Inactivation of STAT3 impaired its nuclear translocation and down regulated the expression of survivin as well as other genes engaged with cell proliferation and survival that are targets of STAT3, such as BCL-XL (B-cell lymphoma XL), cyclin D1 and myc, among others. Taken together, all of these effects could drive tumor cells into a state of dormancy in G0 phase as determined by the low expression of Ki167 protein in tumor cells treated with tyrosine isomers. On the other hand, the S-phase arrest might be generated by a different mechanism that up to date, remains speculative. Several factors and conditions (Ruggiero et al., 2012), such as resveratrol, hyperoxia, hydroxyurea, ultraviolet radiation, G-rich oligonucleotides and zidovudine, induce the inhibition of cell proliferation associated with an S-phase arrest, presumably by the activation of an intra–S-phase checkpoint. Different mechanisms for activating this checkpoint have been proposed, including accumulation of cdk2 (cyclin-dependent kinase 1) in its inactive phosphorylated form, downregulation of cdk2, activation of ATM/ATR (ataxia telangiectasia mutated/ataxia telangiectasia Rad 3-related) kinase in response to DNA damage, modulation or inhibition of a replicative helicase activity, and downregulation of cyclin A2.

After these primary effects, apoptosis and autophagy were observed in some of the previously arrested tumor cells.

Previous reports show that STAT3 inhibition induces signs of autophagy (Shen et al., 2012). Moreover, *m-tyr* may be incorporated into eukaryotic proteins via a specific tRNA-dependent pathway, using mitochondrial and possibly cytosolic phenylalanyl-tRNA synthetase (Lang et al., 2001) and elevated *m-tyr* content in proteins may lead to the dysfunction of intracellular signaling and activation of autophagy. To identify *m-tyr*as a novel inducer of autophagy, we exposed tumor cells to *m-tyr* and assessed LC3 lipidation indicated by the conversion of LC3-I into LC3-II (Gueron et al., 2017). Results showed that endogenous levels of LC3-II accumulated upon *m-tyr* treatment. We also examined if autophagosomes were fusing with lysosomes into autophagolysosomes under *m-tyr*, adding bafilomycin A1 (BafA1). BafA1 inhibits vacuolar-type proton adenosine triphosphatases and prevents fusion between autophagosomes and lysosomes, leading to inhibition of LC3-II degradation (Man et al., 2010). Results showed LC3-II accumulation upon exposure of cells to *m-tyr*in the presence of BafA1, strongly suggesting that *m-tyr* induced autophagosome formation and that the autophagic pathway was functional.

TOWARD A NEW VIEW OF CANCER

Surgical extirpation is the mainstay treatment of solid tumors and may be curative when metastatic cells have not already disseminated from the primary tumor (Chen et al., 2011). However, although it is recommended in most clinical cases, tumor removal may entail an undesired side effect: the acceleration of regional and distant (metastases) residual neoplastic disease (Tohme et al., 2017). This effect may account for the disappointingly modest survival benefits observed when surgery is used as a single strategy of cancer treatment. Some investigators have proposed some therapeutic options to limit metastatic growth after tumor removal,

including the use of perioperative (instead of postoperative) chemotherapy, antioxidant agents, immunotherapy, and bio-modulation(Coffey et al, 2003), but to date, the results have not been as promissory as expected.

The elucidation of the phenomenon of CR could contribute to overcome this problem on the basis that the mechanisms underlying CR can be considered similar or identical to those utilized for a primary tumor to limit the growth of its own natural secondary tumor implants generically known as 'metastases'.

However, in the past, CR has usually been rather neglected by researchers and clinicians probably because the idea that a primary tumor may exert inhibitory influences upon distant metastases meant that a tumor had to be considered an integrated, organ-like entity rather than a collection of independent atypical cells. However, there are numerous observations in the literature that support that idea (Ruggiero et al., 1990; Jirtle and Michalopoulus, 1992; Prehn, 1993; Joseph et al., 2004; Glick and Yuspa, 2005; Ruggiero and Bustuoabad, 2006; Demicheli et al., 2008).For example, hepatectomy stimulates mitosis in previously resting hepatocytes that had been implanted ectopically, or nephrectomy stimulates the proliferation (and also the hypertrophy) of the contralateral kidney in the same way that excision of a primary tumor induces mitosis in previously arrested secondary tumor implants. Furthermore, different from bacteria and other unicellular organisms which grow exponentially if nutrients are available, growth of both normal organs and tumors follow a Gompertzian curve that is exponential at first and then it is modified by an exponential decline in rate when they approach to an asymptote. This decline proved to be not caused by failure of blood and nutrients supply or any other artifact of increased size. The only difference between a normal organ and a tumor, apart from the tendency of a tumor to metastasize, seems to be that the plateau size of the normal organ is reached when the organ reaches its full size, while the putative plateau size of the tumor would be larger than is compatible with the host life (Prehn, 1991). In addition, it has been demonstrated in different murine tumors, that mixtures of particular sub-clones tended, in the resulting tumors, to approach reproducible proportions characteristic for that array of sub-clones and that these final proportions were independent of the starting proportions and of the selective pressures favoring each particular sub-clone (Prehn, 1991). This could hardly have been possible if each particular sub-clone were not in some type of communication with the other sub-clones in order to maintain them in a constant proportion despite different selective pressures.

In a recent paper (Ruggiero et al., 2011) we have elucidated the serum factors responsible for the most universal manifestation of CR, as a mixture of *meta-tyrosine (m-tyr)* and *ortho-tyrosine (o-tyr)*, two unnatural isomers of tyrosine that exhibited strong antitumor effects. In subsequent communications (Machuca et al., 2015; Ruggiero et al., 2015; Gueron et al, 2017; Strazza et al., 2017), we could demonstrate that both *m-tyr* and *o-tyr*could inhibit, in both *in vitro* and *in vivo* settings, not only the proliferation of tumor cells derived from tumors that do induce CR but also that of those derived from tumor that do not induce CR, thus widely increasing their therapeutic possibilities.

The most anti-tumor impressive effect of both tyrosine isomers was achieved on the growth of established metastases of both murine and human origin. Most importantly, these anti-metastatic effects were achieved even at very low concentrations and, different from conventional chemotherapythat usually impairs the health of the body, both *m-tyr* and *o-tyr* seemed to exert their anti-tumor effects without displaying any detectable toxic-side effects even at the highest therapeutic dose.

However, more experiments measuring different physiologic variables not only in mice but also in other species such as rats and rabbits, in acute, sub-acute and chronic schedules of *m*-and *o-tyr* administration, will be necessary to demonstrate more accurately their lack of toxic-side effects.

Taken together, all the experiments reported in precedent communications as well as new experiments, that are underway, aimed to elucidate the molecular basis of the inhibitory effects of *m-* and *o-tyr* on tumor cell proliferation, could help to develop new and less harmful means of managing malignant diseases, especially by controlling the growth of metastases after the removal of a primary tumor, or after other surgical injuries or stressors that have been claimed to promote the escape of metastases from dormancy. We feel that this issue might be of critical importance for experimental oncology and, especially for patients affected by malignant diseases.

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