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Mini-review

Concomitant tumor resistance

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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. This phenomenon has been described in human and animal systems and it can be generated by both immunogenic and non-immunogenic tumors. The relevance of CR to the 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, suggesting that a primary tumor may exert a controlling action on its metastases which could be considered as secondary tumor implants developed spontaneously during the primary tumor growth. A more profound understanding of the different mechanisms claimed to be associated with the phenomenon of CR could contribute to develop new and more harmless means to manage malignant diseases, especially by limiting the development of metastases that arise after resection of primary tumors or after other stressors that may promote the escape of metastases from dormancy.

1. Introduction

1.1. Definition and historical background

The phenomenon of concomitant tumor resistance (CR) is the one by which a tumor-bearing host inhibits or retards the growth of secondary tumor implants. It was first described by Ehrlich [1] but, apart from a few isolated papers [2,3] this phenomenon remained virtually forgotten for about 60 years until it was rediscovered by Gershon and others in the 1960s [4–7]. In 1967, Gershon et al. [6] showed that in hamsters bearing a primary spontaneous lymphoblastic lymphoma, more than 10^5 times more tumor cells were inhibited when implanted as a secondary tumor than was required to produce tumors in normal control animals. In 1969, Lausch and Rappe [7] made a similar observation in hamsters bearing a dimethyl-benzanthracene-induced tumor. Since that moment on, some groups have studied this phenomenon mainly using experimentally-induced and spontaneous tumors growing in inbred rats and mice [8–10]. However, CR has attracted much less attention than 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. For a comprehensive review of the literature prior to 1983, see the excellent work of Gorelik [8].

Resistance of cancer patients to re-inoculation of autologous tumor cells was originally described by Southam [4] and Brunswig et al. [5]. In their experiments, tumor cells were obtained from patients with cancers of the ovary or uterus and autologous tumor cells were inoculated at determined sites on the anterior region of the thigh. The results showed that the anti-tumor resistance to auto-transplantation was more profound in patients with localized cancer than in those with regional or distant metastases. More recently, Kaya et al. [11] and Demicheli et al. [12] found convincing evidence of the presence of CR in patients with osteosarcomas and breast cancer, respectively.

Concerning the relevance of CR with the mechanisms of metastases control, it has been observed that the removal of murine and human tumors may be followed by an abrupt increase in metastatic growth [13–20], suggesting that, upon certain circumstances, a primary tumor exerts a controlling action on its metastases which could be considered as secondary tumor implants developed spontaneously during the primary tumor growth.

There are, at least, two possible explanations for the fact that relatively few groups have systematically studied the phenomenon of CR despite its putative importance for cancer research. In the first place, the study of CR implies the search for anti-tumor mechanisms that depend on the presence of a primary growing tumor; this approach does not seem to have been attractive and promissory for many. In the second place, the study of CR presents some

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methodological problems. In effect; the long latency period of relatively small secondary tumor implants may signify that the animals may die from the primary tumor before the secondary tumor has had a chance to appear. On the other hand, a very large secondary tumor implant may overcome the effect of CR precluding its observation. In consequence, it is necessary to find a balance between the primary tumor volume and the size of the second tumor inoculum. These variables should be studied for each individual tumor since the growth behavior of tumors may vary to a great extent.

2. CR and the inhibition of metastases by the presence of a primary tumor

Local recurrence and especially the metastatic growth is 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), often fall below 15% [21]. Taking into account that the growth of tumor cells re-inoculated into animals bearing a primary tumor mimics the situation that is observed during metastases formation, the understanding of the mechanisms underlying the phenomenon of CR may help to understand the mechanisms responsible for the growth-inhibition of metastatic cells in the presence of a primary tumor. This knowledge could have a significant impact in the management of the malignant diseases.

Many experiments aimed to evaluate CR in animals bearing a subcutaneous (s.c.) growing primary tumor were carried out by re-inoculation of tumor cells implanted by the s.c., intra-muscular (i.m.) or intra-foot pad routes and the intensity of CR was determined by comparing the volume of these secondary tumor implants with that of controls [8,10]. However, probably the best strategy to demonstrate the effect of a primary s.c. tumor on the growth of experimental metastases is the re-inoculation of tumor cells by the intra-venous (i.v.) route. In that case, the intensity of CR generated by the primary tumor would be determined by comparing the number and size of metastatic foci in the lungs of these tumor-bearing mice with those in controls. Different experiments have demonstrated such anti-metastatic effect in mice bearing immunogenic and non-immunogenic tumors [8,16,17].

3. A corollary of CR: acceleration of metastatic growth after primary tumor removal

3.1. Experimental evidence

In experimental settings, accelerated growth of spontaneous metastases following excision of the primary tumor, was described almost a century ago by Tyzzer [22]: he observed that, although the surgical removal of a primary murine tumor prolonged the survival of mice, the size of developed metastatic nodules was larger than in mice bearing the primary tumor. Similar results were obtained by Tadenuma and Okonogi [23]. In the last 50 years, these pioneer experiments were confirmed and extended by different groups by studying the growth of spontaneous and experimentally-induced metastases in tumor-bearing and tumor-excised hosts [8,15–17,24–26]. A rather general pattern derived from these experiments has been reviewed previously [8,15] and can be summarized as follows. The outcome of the removal of a subcutaneous metastatic tumor was dependent on the size of the local tumor removed. When small tumors were surgically excised, the lungs were left with very few metastatic cells as compared with the number in the lungs of tumor-bearing mice 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 mice exceeded the growth of the fewer cells existing in the lungs of the tumor-excised mice. At this stage, tumor excision significantly prolonged the survival of mice. When medium-sized tumors were removed, an *equilibrium* could be reached between the effect of suppression exerted by the primary tumor and the shedding of potentially metastatic cells. In consequence, the total mass of proliferating metastatic cells was similar in both tumor-bearing and tumor-excised mice because although tumor-excised mice displayed fewer lung metastatic foci, each focus was of a larger size. At this stage, tumor removal still – although modestly – prolonged the survival of the operated mice, presumably because even though both metastatic lung 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 level of proliferating metastatic cells and larger metastatic nodules than those present in tumor-bearing mice, were observed. At this stage, tumor excision resulted in a significantly reduced survival of the operated mice.

3.2. Clinical evidence

In clinical settings, an accelerated growth of metastases following tumor resection has been suspected by decades [27]. However, to definitively demonstrate that effect, studies comparing metastatic growth in patients with non-excised tumors (expectant management) with those after tumor resection (surgical management) should be performed. Although these studies are not frequent because surgery is one of the primary treatment modalities for solid cancers, some of them are available in the literature. For example, Iversen et al. [28] 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. [29,30] 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 fourth year after surgery followed by a second peak at the eighth year. Similar patterns of tumor recurrence after mastectomy were observed by other investigators [31], suggesting 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 disease) at diagnosis, has highlighted the problem of CR in human cancer [32]. 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 theoretical disadvantage of surgery is based on the fact that removal of the primary tumor can promote the progression of metastases. Up to date, the clinical studies aimed to solve this controversy showed that tumor removal may improve the survival in patients with stage IV but only in those displaying small primary tumors and limited metastatic load. When larger primary tumors and more metastatic load are present, surgery is not recommended [32]. These clinical results are in agreement with the above referred experimental data

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showing that primary tumor removal can improve or impair the survival of tumor-bearing mice depending on the primary tumor volume and the number of metastatic foci present at the time of surgery.

Although in many other cancers it has not been possible to definitively demonstrate the enhancement of regional and distant (metastases) residual tumor growth after primary tumor removal because of the lack of control non-operated patients, a significant body of evidence accumulated for the last 40 years, has pointed in that direction. For example, Sugarbaker et al. [13] 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 postoperatively, numerous subcutaneous nodules as well as visceral metastases appeared. In the same way, partial spontaneous regression of a primary melanoma is actually a bad prognostic sign (33). Lange et al. [14] reported a study of eight patients who underwent cytoreductive surgery for testicular cancer: in each case, tumor cytoreductive surgery 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 that were unapparent at the time of surgery. Similar findings in patients with epithelial ovarian cancer [34] led to some investigators to urge caution with respect to cytoreductive surgery [34,35].

The above clinical studies together with similar investigations carried out with patients affected by similar or other malignancies strongly suggest that sudden acceleration of metastases may be the undesired outcome of surgical removal of many common human malignancies such as primary melanomas, osteosarcomas and breast, testicular, ovarian, lung, colorectal and bladder carcinomas [13,14,20,27–31,33–39].

4. The reciprocal of CR: concomitant enhancement (CE)

Although the phenomenon of CR has been observed in many experimental and clinical systems, on the other hand, the phenomenon of concomitant enhancement, by which the presence of a primary tumor can stimulate the growth of its metastases, has also been observed [40–42]. In effect, some years ago, Ando et al. [40] found that in mice bearing a spontaneous fibrosarcoma growing s.c. in the hind leg, the number of experimental lung metastases developed after the re-inoculation of tumor cells by the i.v. route, was actually higher than that in control mice. However, in the same mice, the growth of tumor cells re-inoculated i.m. at a distant site from the primary tumor was completely prevented, demonstrating that both CR and CE phenomena could co-exist in the same mice. Similarly, both resistance and susceptibility of tumor-bearing mice to the i.v. tumor challenge was also demonstrated by Janik et al. [41]. More recently, McAllister et al. [42] and Elkabets et al. [43] showed that 2 out of 5 human tumor lines growing s.c. in nude mice, could promote or instigate the growth of otherwise indolent tumor cells, experimental lung micrometastases and tumor surgical specimens, implanted or located at distant sites from the primary tumor. This systemic instigation was associated with the release into the circulation by the primary tumor of osteopontin and other still unidentified instigator-factors. These factors would activate and mobilize into the circulation some types of still non-well known stromal cell precursors from the bone marrow, thereby making them available for recruitment by otherwise non (or poorly) growing tumor cells causing their vigorous growth. In clinical settings, few putative examples of CE have been reported. Most of them have been related to suspected regressions of hepatic and/or pulmonary metastases following nephrectomy for renal cell carcinoma [44–47].

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 tumor relative to that of the smaller one, with high ratios rendering inhibition and low ratios inducing stimulation of the secondary tumor. However, in our experience [48], the magnitude of this stimulatory effect, whenever it is present, proved to be rather modest as compared with the magnitude of the inhibitory effect produced by CR.

In consequence, taken together, the available experimental and clinical evidence suggest us that CR would be more likely than CE to govern the behavior of commonly occurring human tumors.

5. CR-like phenomena beyond the primary-secondary tumors relationship

As referred above, metastatic inhibition or restriction in the presence of a primary tumor can be considered as a particular case of CR which, in turn, may be a particular case of a more general biological phenomenon as far as embryonal masses of tissue (rather like a primary tumor mass) can also restrain the growth of tumors implanted in teratoma-bearing and pregnant mice [49,50]. CR-like phenomena can also be associated with normal organs. For example, hepatectomy stimulates mitosis in previously resting ectopic implants of hepatocytes in the same way that excision of a primary tumor induces mitosis in previously arrested secondary tumor implants [51–53]. Furthermore, a state similar to CR can be developed in organisms infected with parasites or bacteria that are resistant to a second challenge with the same agent. For example, when adult forms of schistosomes were transferred into normal monkeys and two weeks later they were challenged with cercaria, all the animals survived and none showed signs of illness. In contrast, all the control monkeys became ill and died a few weeks later. Curiously, egg production from established adult worms persisted during the destruction of the challenge re-infection, in the same way that tumor cells from the primary tumor continue to grow while the same cells placed in a secondary implantation site are inhibited [8]. Similarly, organisms infected with bacteria such as Salmonella typhimurium or Salmonella enteritidis can be resistant to the re-infection with a second challenge with the same agents [8]. It is unknown whether, besides the obvious differences, a common mechanism underlies, at least in part, the CR and CR-like phenomena described above.

6. Mechanisms proposed to explain the phenomenon of CR

Different hypothesis have been proposed to explain the phenomenon of CR.

According to the immunological hypothesis, the growth of a tumor generates a specific anti-tumor 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 immunologic rejection of allogeneic tumors in naive mice or immunogenic syngeneic tumors in previously immunized animals. The immunological hypothesis was originally proposed by Bashford et al. [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 [10,54]. However, it does not provide a satisfactory explanation for the fact that CR has also been observed in association with spontaneous murine tumors of weakly or non-detectable immunogenicity [8,51,55].

As for non-immunological explanations, basically two hypotheses have been formulated. Ehrlich [1] and Tyzzer [22] believed that nutrients essential for tumor growth are consumed by the primary

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Table 1
Origin, level of immunogenicity and intensity of concomitant tumor resistance induced by 17 murine tumors of different histological type.

Tumor	Origin	Immunogenicity	Concomitant tumor resistance	
			1° Peak	2° Peak
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]	Strong	High	High
MNU-MPA[3]	Induced by MNU + MPA[b]	Moderate	Moderate	Moderate
MC-B[2]	Induced by MC[a]	Moderate	Moderate	Moderate
MNU[3]	Induced by MNU[c]	Weak	Low	Moderate
M3[3]	Spontaneous	Weak	Low	Moderate
MM3[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 foreign body[f]	Undetectable	Absent	Moderate
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

[1] Lymphoma.
[2] Fibrosarcoma.
[3] Carcinoma.
[a] MC = methylcholantrene.
[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] Foreign body = glass cylinder s.c. implanted.

tumor, making it difficult or impossible for a second implant to develop (atrepsis theory). A support for the atrepsis theory is associated with the fact that a progressive tumor is a trap for glucose, nitrogen and other nutrients [8]. 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 [8]. 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 conditions for the proliferation of re-inoculated tumor cells (secondary tumor implant) can not be as favorable as in control animals.

Others [8,51,56–58] have postulated that tumor cells of the primary tumor produce – or induce the production of – anti-proliferative non-specific substances or anti-angiogenic molecules which suppress or limit – directly or indirectly – 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 Nakahara and Fukuoka in their concept of cancer toxohormone, whose circulating concentration should rise with increased tumor mass [59]. More recently, the concept of a substance associated with the phenomenon of CR was re-inforced by the work of Folkman et al. 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 38 kD protein called angiostatin [57].

Taken together, 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 25 years, our group, working at the National Academy of Medicine of Buenos Aires, Argentina, has studied the phenomenon of CR associated with the growth of 17 murine tumors with widely different degrees of immunogenicity, in an attempt to integrate the different hypotheses into a coherent picture (Table 1).

Our results [10,51,60–62] describing two temporally separate peaks of CR during primary tumor growth may explain many apparently contradictory results reported by different authors throughout the years [3,8,10,54] which, in our opinion, were related to the different stages of tumor growth at which each of these authors looked for CR and to the different characteristics of both peaks. In effect, the first peak was observed when the primary tumor was small (<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 cytotoxic antibodies and cell-mediated cytotoxicity against the tumor.

On the other hand, the second peak of CR was induced by both immunogenic and non-immunogenic large tumors ($\geqslant$2000 mm^3); it was not tumor-specific and was thymus-independent as it was exhibited in both euthymic and nude mice and it did not correlate with tumor immunogenicity. The inhibition of the secondary tumor in the presence of a large primary tumor was neither associated with a massive or focal necrosis nor with any host cell infiltration, but with the presence of non-infiltrating tumor cells (dormant tumor) located at the inoculation site between the skin and the muscular layer [62].

Some years ago, an intermediate peak of CR was reported to be associated with a particular type of mid-sized tumors (1000–1500 mm^3) that restrain secondary tumors indirectly, by limiting tumor neo-vascularization [57].

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, has remained an enigma for many years.

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In former studies, we demonstrated that the second peak of CR correlated with the activity of a serum factor(s), different from antibodies or complement, that inhibited the *in vitro* and *in vivo* proliferation of tumor cells. When this serum inhibitory activity was absent – the only two cases were mice bearing the highly metastatic C7HI and MM3 mammary adenocarcinomas – the second peak did not appear. These results suggested a direct correlation among the second peak of CR, the capacity to restrain metastatic growth and the titer of serum growth inhibitory activity. Furthermore, lung metastases produced by C7HI and MM3 tumors 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 [16,17]. We have also demonstrated [17] that this serum factor can also inhibit the *in vitro* proliferation of endothelial cells suggesting that it can also be considered an anti-angiogenic factor. However, its capacity to inhibit endothelial cells proved to be significantly lower that that observed on tumor cells suggesting that its main (although not the unique) anti-tumor effect would be directed on the proper tumor cells.

Partial characterization of this inhibitory activity was previously carried out in our laboratory, rendering a heat, acid and alkali resistant factor of low molecular weight apparently unrelated to other well characterized growth-inhibitory molecules such as interferons, TNF-α, TGF-β, angiostatin and endostatin, taking into account the larger molecular weight of the latter and other physical and biological properties [10,17,51,61].

However, despite these efforts, the origin and chemical nature of that factor remained elusive for years, as well as the paradoxical question concerning why such a factor could inhibit the proliferation of a secondary tumor but not of a large primary one composed of the same type of cells.

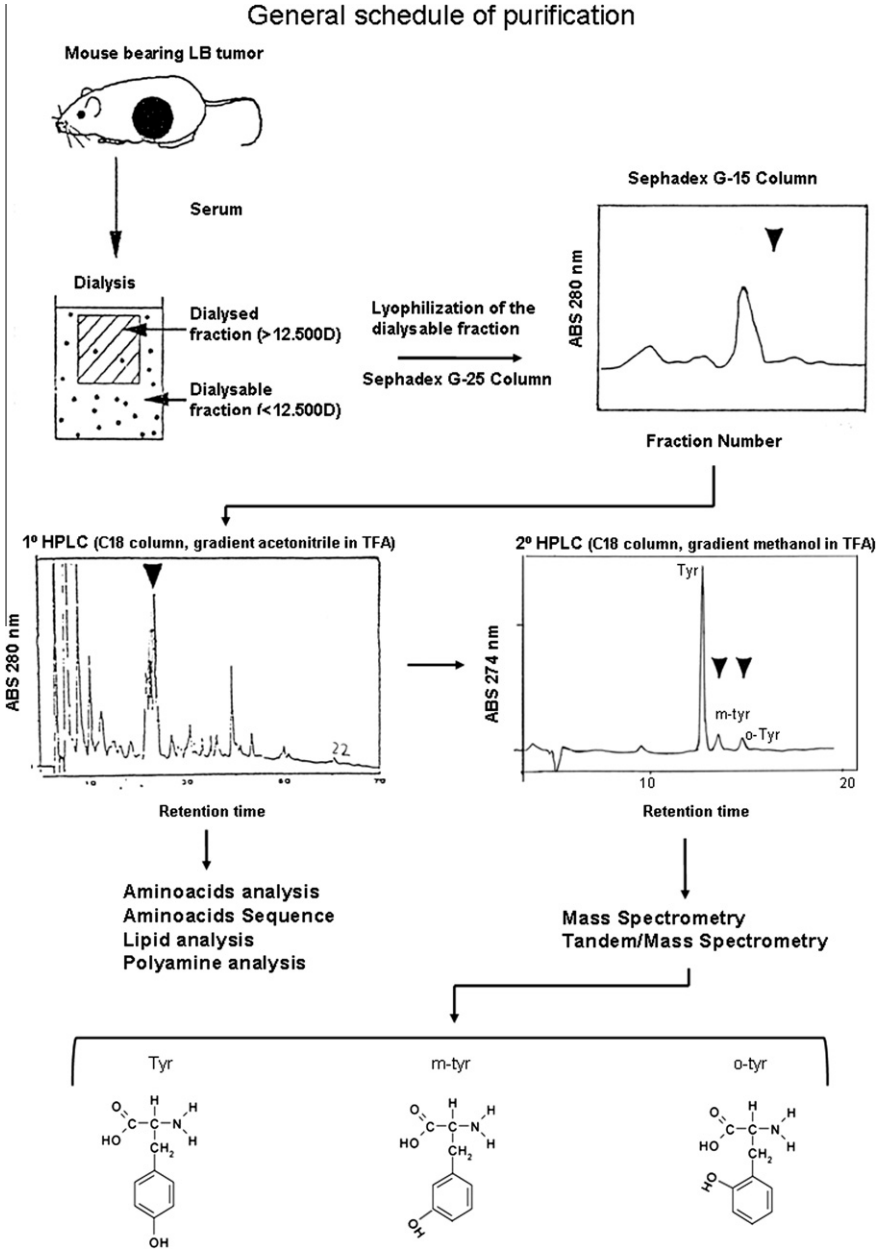

Fig. 1. General schedule of purification of the anti-tumor serum factor(s) associated with the phenomenon of concomitant tumor resistance (CR). The arrow-head (▼) indicates the fractions with anti-tumor activity through the different steps of purification. Anti-tumor activity produced by meta-tyrosine (m-tyr) was about 10 times more robust than that produced by ortho-tyrosine (o-tyr). Conventional tyrosine (Tyr) did not produce any anti-tumor effect. TFA. trifluoroacetic acid.

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7. New discoveries

7.1. Tyrosine isomers mediate the most universal manifestation of CR

In a recently published work [63], starting from mice bearing a non-immunogenic lymphoma (called LB), that produces the strongest second peak of CR among all our tumor models, we have reported the origin, isolation and identification of the serum factor(s) associated with the phenomenon of CR. We have also reported its biological anti-tumor activity and the putative mechanisms of tumor inhibition.

The task of characterization of this factor(s) was long and difficult due to the very low concentration of the active molecule(s) and to the overwhelming amount of tyrosine present in the purified anti-tumor serum fraction, which masked the existence of other molecules and considerably retarded the process of characterization. The elucidation of this puzzle was achieved when, after several steps of purification (see Fig. 1), minimal amounts of meta-tyrosine (m-tyrosine) and ortho-tyrosine (o-tyrosine), two isomers of tyrosine that it is thought to be absent from normal proteins, were finally detected together with tyrosine using high resolution ion-electrospray mass (MS) and tandem mass (MS/MS) spectrometry. M- and o-tyrosine were identified as responsible for 90% and 10%, respectively, of the total anti-tumor activity, as demonstrated by *in vitro* and *in vivo* experiments on the growth of LB and other two murine tumors (MC-C fibrosarcoma and CEI epidermoid carcinoma) that induce CR and on the growth of established spontaneous metastases generated by a highly metastatic mammary adenocarcinoma (C7HI) that does not induce CR but is sensitive to the CR induced by other tumors. The tumor inhibitory effects produced *in vitro* by m- and o-tyrosine were detectable rapidly after 8–18 h in culture even at low (micro-molar) concentrations and those produced *in vivo*, were observed – without exhibiting any toxic side-effects – not only on tumor implants but also on growing vascular (s.c.) and avascular (ascitic) tumors, suggesting that they may have therapeutic potential based on a direct effect on tumor cells rather than an indirect effect on tumor vascularization, However, as suggested by previous experiments [17], an additional inhibitory effect of m- and o-tyrosine on angiogenesis can not be discarded.

The inhibition exerted by m- and o-tyrosine on tumor growth mimics the inhibition produced by CR. In both cases, tumor inhibition was associated with the presence of a high proportion of cells in G0, a decrease in G2-M phases and an increase of the S phase, considered the consequence of an S phase arrest. In addition, both a secondary tumor inhibited by CR and a tumor inhibited by exogenous injection of m-tyrosine, could rapidly reassume their growth when transplanted in a normal mouse or when treatment with m-tyrosine was interrupted, respectively.

The inhibitory effect produced *in vivo* and *in vitro* by m-and o-tyrosine on tumor cell proliferation was counteracted by phenylalanine and, at less degree, by glutamic acid, aspartic acid, glutamine and histidine but neither by tyrosine nor by the remaining protein amino acids.

7.2. The central paradox of CR

The central paradox of CR, that is, the inhibition of secondary tumor implants together with the progressive growth of the primary tumor, has remained unsolved for more than a century. To account for this problem we demonstrated that, as a primary tumor grows, relatively large amounts of most amino acids, including those that counteract the inhibitory effects of m- and o-tyrosine (phenylalanine, glutamic acid, aspartic acid, glutamine and histidine), are accumulated in the tumor microenvironment while at distant sites, such as sites of putative secondary tumor implants, the content of amino acids is significantly lower.

On this basis, we have suggested that a secondary tumor can be inhibited by circulating m- and o-tyrosine at the same time as the primary tumor can be protected, at least in part, from their inhibitory effects by those counteracting amino acids and thus could continue to grow. This suggestion seems to reconcile the two major non-immunological interpretations of CR that have been advanced in the past: the hypothesis of anti-proliferative factors and the atrepsis theory [1,8,22,51,56,57]. In effect, the postulation of serum m- and o-tyrosine as responsible for the inhibitory effect generated by a primary tumor on the growth of secondary tumor implants reminds the hypothesis of anti-proliferative factors. However, the mere presence of inhibitory factors such as m-and o-tyrosine is not enough to explain why the primary tumor can grow while the secondary one can not. On the other hand, the different concentration of amino acids at the site of the primary tumor as compared with other parts of the organism, reminds the atrepsis theory because according to this theory, the primary tumor accumulate elements that would allow it to grow and whose lack at distant sites from the primary tumor, would prevent a second tumor to grow. However, while in the atrepsis theory these elements are nutrients that would directly stimulate the primary tumor growth, in our postulation they would allow the primary tumor growth by counteracting the effect of circulating inhibitory factors. Some years ago, Prehn [64] anticipated this interpretation suggesting that CR could best be explained by the competitive interaction of two opposing – and up to that time uncharacterized - influences, a local slowly diffusible, tumor-facilitating environment, that would be counteracted by circulating inhibitors.

7.3. Origin of tyrosine isomers and putative mechanisms of tumor inhibition

Up to date, m- and o-tyrosine have been studied, almost exclusively, as markers for oxidative damage associated with abnormal proteins detected in the blood of animals subjected to cardiac ischaemia–reperfusion injury, mitochondria of exercised animals, atherosclerotic tissue of diabetic primates, aging lens of human beings, etc. [65].

Most studies have assumed that m- and o-tyrosine are generated post-translationally when L-phenylalanine present in proteins is exposed to hydroxyl radicals during oxidative damage. However, it has recently been suggested that oxidized amino acids, such as m- and o-tyrosine, might also be generated from free amino acids that could be subsequently incorporated into proteins during synthesis [65,66]. We previously observed that the serum anti-tumor activity attributed to m- and o-tyrosine was strongly inhibited by agents that reduce the number of myeloid-derived suppressor cells (MDSC) and the oxidative damage, and that, in tumor-bearing mice (including the LB tumor model used in our previous work [63]) and in some cancer patients, MDSCs that produced large amounts of reactive oxygen species (ROS) accumulate progressively in circulation [63,67–71]. On this basis, we suggested that free m- and o-tyrosine present in serum from tumor-bearing mice would be produced, at least in part, when circulating molecules of phenylalanine are oxidized by hydroxyl radicals released by MDSC. In the last few years, the role of the bone marrow-derived MDSC in tumor biology has been highlighted by different investigators which demonstrated that MDSC would be a major component of the immune-suppressive network observed in tumor-bearing hosts. In these mice, MDSC are present not only in circulation but also in peripheral lymphoid organs and at the proper tumor site. The hypoxia present at the tumor site, via the hypoxia-inducible factor (HIF), seems to regulate the conversion of MDSC to non-specific immune suppressors and their preferential differentiation to the highly immune suppressor tumor-associated macrophages (TAM) [71,72]. In consequence, our findings concerning the origin of m- and o-tyrosine - apparently

responsible for the most universal manifestation of CR -, add a new putative role played by MDSC on tumor growth.

Very few studies have previously reported anti-proliferative effects mediated by m- and o-tyrosine. Gurer-Orhan et al. [65], while studying alternative mechanisms for oxidative stress and tissue injury during aging and disease, showed that free m-tyrosine and o-tyrosine were toxic to chinese-hamster ovary (CHO) cells when these cells were incubated *in vitro* with m-or o-tyrosine for 7–10 days. In the same way, Bertin et al. [66], while studying the development of more environmentally friendly weed management systems, demonstrated that the unusual ability of many fine fescue grasses to outcompete or displace other neighboring plants was based on the phytotoxic properties of their root exudates and that more than 80% of the active fraction was m-tyrosine. Both authors hypothesized that one potential cytotoxicity mechanism could involve mischarging of tRNA and consequent misincorporation of these unnatural isomers of tyrosine into cellular proteins based on their structural similarities with phenylalanine or tyrosine. In turn, this misincorporation could cause structural disruption in proteins or could interfere with the functions of key enzymes such as DNA polymerase which might lead to errors in DNA replication and long-term consequences such as impaired cellular viability.

The mechanism of misincorporation into cellular proteins, claimed to be associated with long-lasting cytotoxicity effects on mammal and plant normal cells, could also be invoked to explain the short-lasting anti-proliferative effects of m- and o-tyrosine on tumor cells described in our previous paper [63]. Although this alternative is possible, some of their anti-tumor effects might start before such misincorporation in proteins had a chance to occur. This is suggested by the rapid reversion of those effects, by the counteracting effects of amino acids (other than phenylalanine) that lack any obvious structural similarity with m- and o-tyrosine and in consequence with less possibilities to compete for the same tRNA, and by molecular analysis that showed that the anti-tumor effects mediated by m- and o-tyrosine were mediated, at least in part, by a very early inhibition of MAP/ERK signaling pathway which would drive tumor cells into a state of dormancy in G0-phase through a rapid decay of p-STAT3 [63]. Other mechanisms, putatively involving the activation of an intra-S phase checkpoint, would also inhibit tumor proliferation by accumulating cells in S-phase. Speculations concerning the intimate mechanisms by which a partial inactivation of p-STAT3 and an activation of an intra-S phase checkpoint could drive tumor cells into a state of dormancy have been reported elsewhere [73]. Whatever these intimate mechanisms, it is provoking that the same molecule, m-tyrosine, has been preserved throughout the evolution as an anti-proliferative factor in two different biological kingdoms.

8. Conclusions and perspectives

Surgical extirpation is the mainstay treatment of solid tumors and may be curative when metastatic cells have not already disseminated from the primary tumor. However, although recommended in many clinical cases, tumor removal may entail an undesired side-effect: the acceleration of regional and distant (metastases) residual neoplastic disease. Such effect may account for the disappointingly modest survival benefits observed when surgery is used as a single strategy of treatment. Some therapeutic options after tumor removal have been proposed to limit metastatic growth. They include the use of peri-operative (instead of post-operative) chemotherapy, antioxidant agents, immunotherapy and bio-modulation [35] but, up to date, the results were not as promissory as expected.

The elucidation of the phenomenon of CR could contribute to overcome this problem, but in the past CR has usually been 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 [27,51–53,64,74,75]. For example (as also referred in a precedent item), hepatectomy stimulates mitosis in previously resting hepatocytes that had been implanted ectopically, in the same way that excision of a primary tumor induces mitosis in previously arrested secondary tumor implants [51–53]. 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 with the approach to an asymptote [76,77]. 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. Some years ago, Prehn [78] typified this situation indicating that "perhaps one could say that a malignant tumor of the mouse simulates, in the pattern of its growth curve, a normal organ in a rabbit or possibly, in extreme cases, an elephant!" 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 [78–81]. 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.

Along this new conceptual model of cancer, a more profound understanding of the different immunologic, anti-angiogenic and m- and o-tyrosine-dependent mechanisms associated with the phenomenon of CR could contribute to unveil some of the control mechanisms of malignant and normal cell proliferation and to develop new and more harmless means to manage malignant diseases. Depending on the tumor involved, it is possible that its sensitivity to the different mechanisms associated with CR may be different. In consequence, the study of each particular case will be necessary to design the best strategy aimed to control the growth of metastases after the removal of a primary tumor or after other surgical injuries or stressors that may promote the escape of metastases from dormancy [27,82–85].

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