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All-trans-retinoic acid counteract the tumor-stimulating effect of hepatectomy and increases survival of rats bearing liver metastases

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Background: We previously demonstrated a stimulating effect of hepatectomy on residual tumor cells after resection of liver metastases. The aim of this study was to analyze the effect of all-trans-retinoic acid (ATRA) on the protumor effect of hepatectomy and survival of hepatectomized rats bearing liver metastases. We also explored whether ATRA interfered with the tumor promoting effect of hepatotropic growth factors (GFs).
Methods: The *in vitro* effect of ATRA on proliferation of S4MH rhabdomyosarcoma tumor cells was assessed when cultured with laparotomized or hepatectomized rat serum (HRS), or in the presence of GFs (hepatocyte growth factor, insulin growth factor 2, Platelet Derived Growth Factor (PDGF)-BB, and vascular endothelial growth factor). For the *in vivo* studies, rats were partially hepatectomized on day 10 after metastasis induction, one group being treated with ATRA from day 7 to 14, and a second receiving cyclophosphamide (CY; on days 10 and 14) alone or with ATRA. We determined the size and number of liver and lung metastases. Finally, we analyzed the effect of treatments on rat survival.
Results: Hepatotropic GFs increased cell proliferation in a similar manner to HRS. *In vitro*, ATRA blocked the protumor effect of both HRS and GFs. *In vivo*, ATRA reduced the size and number of liver and lung metastases, and significantly increased rat survival. Furthermore, adding ATRA to CY significantly increased survival compared with CY alone.
Conclusions: In our model, ATRA minimizes the tumor-stimulating effect of hepatectomy, reducing the number and size of liver metastases and improving survival. The results suggest that the ATRA may be useful for blocking the growth-promoting effect of hepatotropic GFs released after liver metastasis resection.

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1. Introduction

Liver metastases are a serious clinical challenge, since up to a third of all the metastasizing cancers involve the liver [1], and the progression of these metastases is one of the main causes of death in cancer patients. At present, partial liver resection of localized metastases provide the best results in terms of long-term survival, 5-y survival rates of up to 47% being reported [2].

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Although surgical resection is the current paradigm of treatment, this procedure is only potentially curative, as up to 80% of patients experience relapse within 2 y of undergoing surgical resection [3,4]. In the last decade, neoadjuvant chemotherapies have been designed to reduce the volume of liver metastases in order to increase the number of resectable patients [5]. In any case, recurrence is still the main complication of metastasectomy [6].

There are several factors that must be considered regarding recurrence of liver metastases. First, seeding of cancer cells in the liver is a wide spread phenomenon, many of them remaining silent, whereas others grow asynchronously giving rise to various macroscopic metastases. Second, surgical removal of these metastases does affect other cancer cells present in the remnant liver. In fact, we have previously demonstrated the stimulating effect of hepatectomy on residual tumor cells in the liver [7,8], also showing that the proliferative stimulus induced by hepatectomized rat serum (HRS) is twice that induced by laparotomized rat serum (LRS), making it important to analyze the hepatotropic factors specifically responsible for this tumor-enhancing effect. After partial resection, several growth factors (GFs), which are responsible for liver regeneration, are released locally. GFs such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), basic-fibroblastic growth factor (FGFb), insulin growth factor I (IGF-I), and vascular endothelial growth factor (VEGF) have been reported to be associated with tumor progression [9]. All of these GFs could be important stimuli for the aforementioned silent cancer cells, promoting new metastases. In addition, we and other authors have suggested that GFs, such as HGF, EGF, and VEGF induce chemoresistance, significantly reducing the cytotoxic activity of certain common active antitumor agents [10,11].

In the light of the high associated rate of recurrence, it is essential to develop new preventive therapeutic strategies directed to block the effect of GFs released after hepatectomy on cancer cells, while not disturbing wound healing and liver regeneration.

As cell proliferation and differentiation are deregulated in tumor cells, the induction of cell differentiation with retinoids could help to neutralize the protumor effect of GFs. The mechanisms of action underlying the effects of retinoids include not only the activation of nuclear retinoic acid receptors (RARs) and the retinoid x-receptors [12], but also the induction of an increase in Reactive Oxygen Species levels [13] and a direct interaction of retinoids with the glutathione-dependent protein kinase C, a key regulatory enzyme in signal transduction [14]. In relation to this, we have previously analyzed the effect of all-trans-retinoic acid (ATRA), a well-known prodifferentiating agent, on tumor recurrence and metastatic process. This drug was found to reduce the proliferative rate in an *in vitro* and *in vivo* rat model of rhabdomyosarcoma (RMS) [13,15]. Other authors have also demonstrated the effectiveness of retinoids as differentiation inducers in RMS cell lines, suggesting the potential of ATRA for RMS treatment [16]. In addition, it has been described that ATRA, decreasing secretion of some GFs, can reduce proliferative activity of several tumor cell lines, including RMS [17].

Based on the aforementioned data, the aim of this study was (i) to analyze the effect of GFs on tumor cell proliferation of a metastatic RMS cell line with high affinity for liver tissue; (ii) to explore whether ATRA interferes with the tumor-promoting effect of GFs; and (iii) if so, whether it could contribute to increasing survival after partial hepatectomy and cyclophosphamide (CY) treatment, in rats bearing RMS liver metastases.

2. Materials and methods

2.1. *Tumor cell culture*

The study was carried out on a poorly differentiated and highly metastatic RMS cell line S4MH, which was selected because of its high affinity for liver tissue. Tumor cells were grown and subcultured in Dulbecco's Minimum Essential Medium (DMEM; Sigma, St. Louis, MO) supplemented with 15% fetal calf serum (FCS), 100 IU/mL penicillin and 100 g/mL streptomycin at 37°C in a humidified, 5% CO_2 incubator.

Exponentially growing cell cultures were used in all experiments. After a short period of exposure to phosphate buffered saline (PBS)/EDTA (2 mM) and centrifuging, the pellet was resuspended in the complete medium plus FCS, and a cell count obtained with a Coulter counter (Coultronics, Margency, France). Viability, as determined by trypan blue exclusion, ranged from 95% to 98%.

2.2. *Chemicals*

ATRA was purchased from Sigma Chemical Co (St Louis, MO). For *in vitro* studies, ATRA was dissolved in 100% ethanol to obtain 10^{-2} M stock solutions, which were stored in the dark at −20°C. The stock solution was diluted in medium to obtain the appropriate final concentration (10^{-6} M). The maximum concentration of ethanol in the culture was <0.1%, and it did not affect cell growth. The culture medium containing ATRA or the solvent was replaced every 48 h. For *in vivo* studies, ATRA was dissolved in ClinOleic (90%) and ethanol (10%) to obtain a 2 mg/mL concentration of the drug. CY was obtained from Sigma Chemical Co and dissolved in sterile physiological saline solutions (0.9% NaCl) adjusted to pH 7.0. GFs were also obtained from Sigma Chemical Co and reconstituted in accordance with their specification sheets.

2.3. In vitro *cell proliferation studies*

S4MH cells were seeded in 24-well microplates at a density of 10^4 cells/well in 10^3 μL of growth medium plus 15% FCS and allowed to attach and grow for 24 h. The cells were then exposed to DMEM supplemented either with 15% FCS or 15% of serum obtained from hepatectomized or laparatomized rats (HRS or LRS, respectively). HRS and LRS were obtained from blood drained from the aorta of WAG/RijCrl rats, which had been subjected (40 h earlier) to either a 40% hepatectomy or a laparotomy. Another set of experiments was carried out in the presence of either HGF (10 ng/mL), VEGF (10 ng/mL), EGF (25 ng/mL), FGF (10 ng/mL), PDGF-BB (10 ng/mL), or IGF-2 (10 ng/mL). The concentration of each GF was chosen on the basis of preliminary studies performed to determine which induced the maximum increase in proliferation (data not shown).

In the experiments with ATRA (10^{-6} M), the growth medium (plus 15% FCS) containing ATRA or the solvent was replaced every 48 h. The *in vitro* effect of ATRA on cell proliferation was analyzed in the presence and absence of the aforementioned GFs. Cell proliferation was measured at 24, 48, and 72 h, using a hemocytometer to count the cells growing in each well. Each assay was repeated three times and all experiments were performed in sextuplicate wells.

2.4. *Animals and liver metastases induction*

All surgical procedures were carried out in 8-wk-old male WAG/RijCrl syngeneic rats (Charles River, Barcelona, Spain) under Nembutal anesthesia (45 mg/kg, intraperitoneally [i.p.]). The animals were kept in cages with free access to water and food (Panlab A-04), with a constant 12-h light–dark cycle. Guidelines for the care of animals kept for experimentation and other scientific purposes [18] were respected at all times.

To induce liver metastases, 25×10^4 tumor cells suspended in 0.5 mL of Hank's solution were inoculated into the spleen, and the organ was placed back into the peritoneum for 5 min to allow for cell migration to the liver sinusoids; then the spleen was excised. Ten days later, a 40% hepatectomy was performed.

2.5. *Experimental series and assessment of antitumor effect*

For *in vivo* experiments, groups of 14 animals were used; half of the animals were randomly allocated to receive the treatments, whereas the other half were only given the solvent (control group). ATRA treatment (5 mg/kg) was administered daily i.p. from 3 d before hepatectomy until the 10th day after this surgery. CY was also administered i.p. in two doses of 50 mg/kg, on days 10 and 14.

To study the antitumor effect of ATRA, alone and in combination with CY, two types of experiments were performed. The first experiment was to determine tumor development by quantifying the number of metastases. In the case of ATRA alone, controls and ATRA-treated animals were sacrificed on day 21 after tumor inoculation, to determine the number of metastases. With respect to groups of rats treated with CY or ATRA + CY, because of the delay in the appearance of metastases, animals were sacrificed on day 50 after tumor inoculation. Macroscopic metastases, in the lungs and in the liver, were assessed as previously described (García-Alonso, 2008). Briefly, the lungs were inflated through a cannula placed into the trachea, and the number of superficial metastases was assessed. The liver was removed, each lobe was sectioned into 4-mm thick slices, and the number of visible metastases was counted. Afterward, the liver samples were embedded in paraffin and serially sectioned (5-μm thick, 100-μm apart) and stained with hematoxylin-eosin. Quantitative information on the size of metastatic foci, expressed as mean surface area, was obtained by using an integrated semiautomatic image analysis system. All these procedures were performed by a "blinded observer."

Second, other groups of animals were used to determine the effect of treatments on the survival of the animals. Besides obtaining survival curves, we compared the median survival time of treated groups (T) with that of control groups (C), expressing this as a T/C value. A T/C value of at least 125% is required to demonstrate activity [19]. In addition, the percentage of long-term survivors (LTS) on day 85 was calculated compared with the total number of rats per group.

2.6. *Toxicity analysis*

Toxicity end-points included body weight loss, hematological toxicity, and serum liver enzyme levels. The animals were monitored daily, starting on day 7 after tumor cell inoculation, to record body weight changes. On days 10, 15, and 22, blood samples were obtained from each animal for measuring hematological parameters (peripheral white blood cells [WBC], red blood cell, and platelet counts) and serum enzyme levels (aspartate transaminase, lactate dehydrogenase, and alkaline phosphatase [AP]).

2.7. *Statistical analysis*

Statistical analysis was performed using GraphPad (San Diego, CA). *In vitro* studies involved more than two groups, so an analysis of variance was used to analyze the results obtained; when analysis of variance showed statistically significant differences ($P < 0.05$), a Newman–Keuls test was carried out to establish the group(s) responsible. As all the results *in vivo* passed the normality test (Kolmogorov–Smirnov Test), a Student *t*-test was used to analyze differences, which were considered significant when $P < 0.05$. Survival results were analyzed using Kaplan-Meier curves and log-rank (Mantel-Cox) test.

3. Results

3.1. *Effect of LRS, HRS, or GFs on the proliferation rate of S4MH cells*

Based on the fact that surgical trauma, such as laparotomy or hepatectomy, induces a release of GFs to serum, HRS and LRS were collected from rats to compare their influence on cell proliferation with respect to FCS under standard cell culture conditions. We observed, at 72 h, that the proliferation of cells cultured in presence of HRS was 1.4 and 2 times higher than that of cells cultured with FCS or LRS, respectively (Fig. 1A).

To analyze the involvement of various GFs in the growth promoting effect of HRS, S4MH cells were exposed to individual GFs (HGF, VEGF, PDGF-BB, IGF-2, FGFb, and EGF). Treatment of cells with these GFs was accompanied by significantly higher proliferation rates than those in control cells. Specifically, exposure to VEGF resulted in proliferation rates 1.4-fold higher ($P < 0.05$) and to each of the other of the GFs studied 1.3-fold higher ($P < 0.05$) than those observed in control cells at 24 h (Fig. 1B). This growth-promoting effect was maintained until 72 h, when a slightly higher rates (1.3-fold higher with respect to controls; $P < 0.05$) were seen with EGF and FGFb, than with the other GFs (1.2-fold with respect to controls; $P < 0.05$).

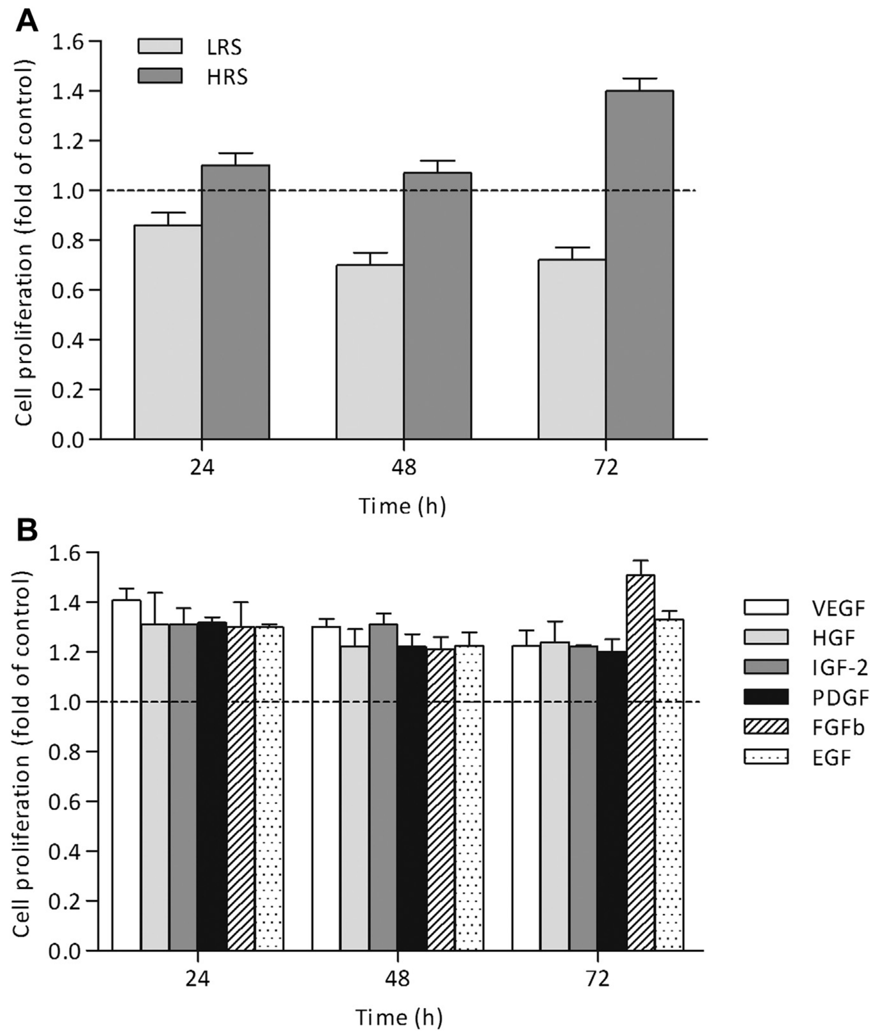

Fig. 1 – Proliferation of S4MH cells cultured in the presence of HRS, LRS or different GFs. (A) HRS exerts a stronger proliferative effect on tumor cell cultures than LRS. (B) All the GFs tested increased cell proliferation when compared with controls.

3.2. *Effect of ATRA on proliferation rate of S4MH cells cultured in the presence of LRS, HRS, or GFs*

Tumor cells were exposed to nontoxic concentration of 10^{-6} ATRA. Consistent with our previous results, with this treatment the growth rate of DMEM + FCS cultured S4MH cells was 1.4-fold lower, at 72 h ($P < 0.01$) [13].

In this work, we first analyzed the effect of ATRA treatment on the proliferation of this tumor cell line cultured with DMEM + HRS or DMEM + LRS. As shown in Figure 2, ATRA resulted in growth rates of S4MH cells significantly lower than in controls in both cases. However, this reduction was larger when the cells were cultured with HRS (2.8-fold) than with LRS (1.9-fold). In fact, ATRA reduced the protumor effect of HRS in such a way that no significant differences in cell number were observed between S4MH cells cultured with HRS or LRS at 24, 48, and 72 h.

Second, we analyzed the effect of ATRA treatment on the proliferation of S4MH cells cultured in the presence of GFs. As shown in Figure 3, ATRA overcame the growth-promoting effect of all GFs. Thus, at 72 h, ATRA abrogated the protumor effect of HGF, IGF-2, PDGF-BB, and VEGF, and no significant differences were observed in the effect of ATRA on cell proliferation in the presence or absence of these GFs. In the case of EGF and FGF, although the effect of ATRA was less pronounced than with the other GFs, it also significantly reduced their protumor activity, and no significant modification in the proliferation rate was found when compared with untreated cells cultured in the absence of GFs, at 72 h.

3.3. *Effect of ATRA alone and in combination with CY on tumor progression and toxicity on hepatectomized rats bearing liver metastases*

Based on our previous *in vivo* results, in which we observed a tumor-enhancing effect secondary to hepatectomy [8], and on the previously mentioned *in vitro* effects induced by ATRA, we then first studied the effect of ATRA on metastasis development in hepatectomized rats bearing liver metastases, in an attempt to determine whether *in vivo* ATRA treatment

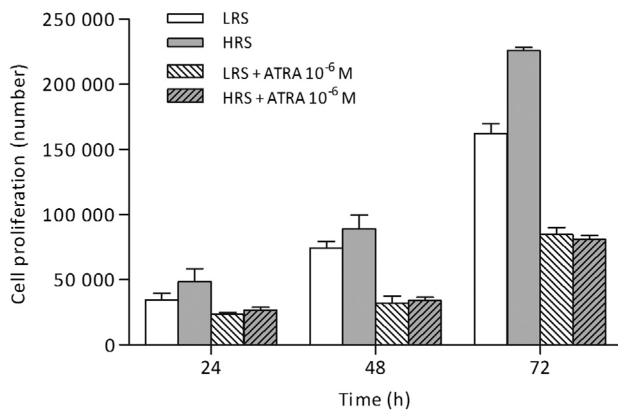

Fig. 2 – Effect of ATRA on S4MH cell proliferation in the presence of HRS or LRS. ATRA addition to cultures blocks the stimulating proliferative effect of both LRS and HRS.

was able to counteract the tumor-stimulating effect of hepatectomy.

To evaluate the effect of ATRA on the liver and lung metastasis development, the number of metastases was recorded in rats previously subjected to 40% hepatectomy. In terms of tumor development, macroscopic liver metastases were found in all animals analyzed on day 21. As shown in Figure 4, there were significantly (2.2-fold) fewer macroscopic metastases in the liver in the ATRA-treated than the solvent (Clin-Oleic)-treated group (3.1 ± 0.7 *versus* 6.8 ± 1.0; $P = 0.005$). There were also fewer metastases in the lungs of ATRA-treated rats (30 ± 12 *versus* 46 ± 10) than those of the controls, but the difference did not reach statistical significance ($P = 0.16$).

In addition, the microscopic study of the liver samples showed that the mean size of the foci, expressed as mean surface area (Fig. 5), was significantly lower in the ATRA-treated group than the control group (0.3 ± 0.1 mm^2 *versus* 1.1 ± 0.3 mm^2; $P = 0.04$).

The aforementioned *in vivo* results obtained with ATRA treatment, prompted us to investigate the effect of this therapy in combination with CY, on metastasis development and overall survival of animals. Although liver metastases could be seen in all untreated animals by day 21, all rats treated with CY were found to have developed macroscopic metastasis by day 50. At this point, the number of liver metastases was 4.8-fold lower ($P = 0.016$) with the combination of ATRA and CY than with CY alone (0.92 ± 0.43 *versus* 4.4 ± 1.3; Fig. 6).

The differences in the number of macroscopic foci and in the size of the microscopic foci observed in the ATRA-treated animals compared with controls correlate well with the significantly longer overall survival observed in this treated group ($P = 0.03$). As shown in Table 1, ATRA treatment resulted in T/C values of 134%. As expected, a marked increase in survival was found with CY treatment, the T/C value being 190% with 33% LTS. Furthermore, in the case of ATRA added to treatment with CY, the life span was significantly longer (T/C values of 228% with 50% LTSs) than with CY alone.

To analyze the possible toxicity of ATRA treatment (alone and in combination with CY) in hepatectomized animals, body weight loss, hematological toxicity, and changes in serum liver enzyme levels were recorded as a means of assessing systemic toxicity. With respect to animal body weight, as expected, a significant weight loss (2.7% ± 1.5) was detected in the hepatectomized control group on day 13 (72 h after hepatectomy), with a recovery to the baseline weight by day 17 (1 wk after liver resection). There were no significant differences in body weight between the ATRA-treated group and hepatectomized control animals. In CY-treated animals a significantly greater weight loss (8.8% ± 1.9) was registered on day 13; in this case, the recovery to the initial weight was delayed until day 21. However, adding ATRA to CY did not modify the body weight with respect to the group treated with CY alone.

Regarding hematological toxicity, the hepatectomy did not induce significant changes in the WBC or platelet count, and only a slight decrease in the number of red blood cells was detected (10% and 13% reductions on days 15 and 22, respectively; $P < 0.05$). In comparison to hepatectomized control group, no significant differences in platelet and red blood cell count were observed in the ATRA-treated animals, whereas WBC was significantly lower (by 38%) on day 15, with a recovery to normal values by day 22. In the case of CY-treated animals, a significant reduction in red blood cells (14%) and platelets (52%) was noted on day 15, this remaining to day 22; furthermore, a severe drop on WBC count (92% reduction) was seen on day 15, with a partial recovery up to 73% of control values by day 22. There were also significant changes in the three hematological parameters when ATRA was added to CY treatment. Specifically, red cell counts were 21% lower than in the group treated with CY alone, both at day 15 and 22. The decrease in the number of platelets on day 15 was similar to that observed in animals treated with CY alone (57% decrease), but at day 22 the values recovered to the levels in controls. As for WBC count, curiously, after the initial depletion (90% fall at day 15), there was significant leukocytosis (76.6% increase in WBC count with respect to controls) by day 22.

Finally, in relation to serum activities of liver enzymes, no significant changes were found in aspartate transaminase, ALT, or lactate dehydrogenase levels among groups, whereas significantly higher ALP levels were observed on day 22 in ATRA-treated group (244 ± 74 U/L) and ATRA + CY-treated group (199 ± 48 U/L), compared with those of the controls (56 ± 15 U/L).

4. Discussion

Hepatectomy is often the only treatment modality that has the potential to achieve long-term survival in patients with liver metastases. However, the high incidence of recurrence after liver resection observed in clinical experience limits the therapeutic potential of this surgery and the possibility of a cure for these patients. Given this, it is essential to optimize the integration of medical treatment with surgery to improve outcomes in liver metastases.

The demonstration in several experimental tumor models that surgical trauma, and particularly liver resection, can facilitate metastatic spread [7,8,20,21], prompted us to

Fig. 3 – Effect of ATRA on S4MH cell proliferation in the presence of GFs. ATRA inhibits the growth-promoting effect of GFs.

investigate a new therapeutic approach based on the use of ATRA, aiming to minimize the impact of surgical resection on metastatic stimulation. Using the experimental S4MH RMS model of liver metastases, in the present study, we demonstrated that ATRA treatment may effectively reduce the protumor effect of hepatectomy. Other authors have also suggested that the administration of retinoids, and in particular ATRA, could be an effective strategy for differentiation

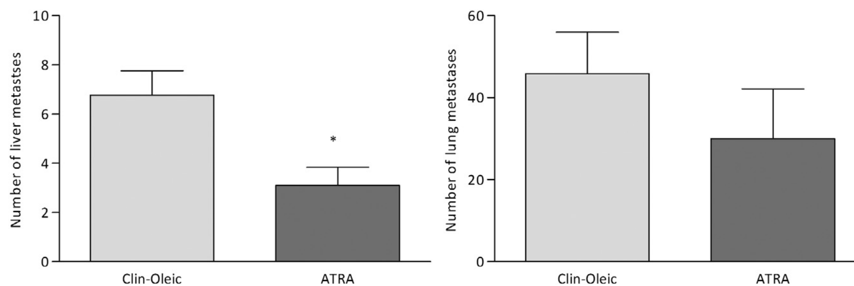

Fig. 4 – Effect of ATRA treatment on number and size of macroscopic liver (left) and lung (right) metastases in hepatomized rats (data referred to day 21). ATRA reduces the number of macroscopic liver metastases ($P < 0.01$). Although differences were not significant, a similar trend was observed in the case of lung metastases.

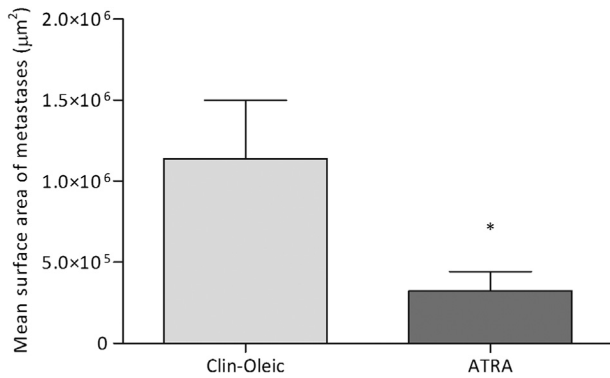

Fig. 5 – Assessment of mean surface area of the liver parenchyma occupied by tumor on ATRA-treated and nontreated hepatectomized rats. The tissue area occupied by tumor was significantly reduced by ATRA ($P < 0.05$).

therapy of lung and hepatic cancer metastasis [22,23], and as a chemopreventive agent against the risk of hepatocellular carcinoma recurrence [24].

In addition, it has been demonstrated that ATRA induces growth inhibition and suppresses the self-renewal of cancer stem cells, which has been associated with downregulation of Wnt/β-catenin signaling [25]. Because of this, it has been suggested that the combination of ATRA and conventional therapy could significantly inhibit tumor growth, metastasis, and recurrence [26].

This antitumor activity of ATRA observed in our study could be explained by the abrogation of the protumor effects of GFs released during surgery. In fact, in the *in vitro* experiments we found that this retinoic acid reverted the proliferative stimulus of serum derived from hepatectomized rats, no differences in proliferation rate being found between cells cultured with LRS or HRS when ATRA was added to cultures.

To analyze the possible influence of molecular mediators released by the surgical trauma of hepatectomy, we analyzed the influence of a wide variety of GFs on proliferative activity

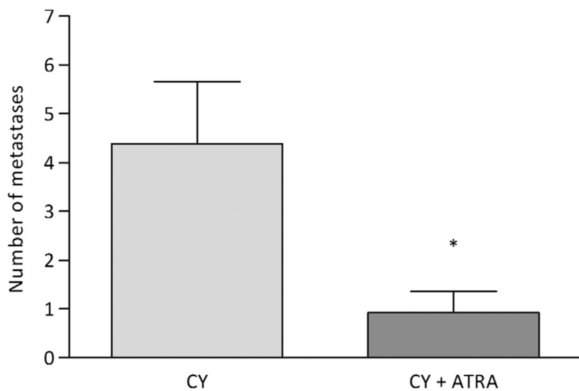

Fig. 6 – Mean number of liver metastases in hepatectomized rats treated with CY alone or in combination with ATRA. The addition of ATRA to CY therapy induces a greater reduction of the number of tumor foci compared with CY alone ($P < 0.05$).

Table 1 – Survival of rats bearing S4MH liver metastases treated with ATRA (alone or in combination with CY).

Treatment	Survival		
	Median (d)	T/C (%)[*]	LTSs (%)[†]
Control	35	–	0
ATRA	47	134	0
CY	66.5	190	33
ATRA + CY	80	228	50

[*] Increase in life span, expressed as a percentage (a value >125 is required to demonstrate activity).
[†] Percentage of LTS on day 85 more than the number of rats per group.

of S4MH cells. All the GFs studied (HGF, VEGF, PDGF, EGF, and FGFb) exerted a similar proliferative stimulus, although the strongest initial effect was found with VEGF. In these specific *in vitro* experiments, we demonstrated that ATRA also completely abolished the growth stimulating effect of all GFs. Other authors have also demonstrated that ATRA treatment slowed RMS cell growth, specifically through a decrease in VEGF secretion [17], which suggests that ATRA could have a possible antiangiogenic effect. Similarly, the inhibition of HGF secretion by ATRA has been also demonstrated in another tumor model [12].

Given the inhibitory effect of ATRA on the GF protumor action observed in *in vitro* studies, we decided to use ATRA as a chemopreventive agent, starting the treatment 3 d before hepatectomy and maintaining it postoperatively to test whether an inhibitory effect is produced also in the *in vivo* situation. We found that this retinoic acid was able to drastically reduce the number of macroscopic metastases, not only in the liver, but also those located in the lung. In addition, we observed that the mean size of the metastatic foci was also significantly smaller, indicating that ATRA is also able to delay the development of remnant metastasis. All of these factors contribute to the increase in overall survival of treated animals.

These *in vivo* effects progressed with no noticeable side effects, only a transient reduction in WBC count and an augmentation of ALP levels being observed. This ALP increase in response to ATRA has been also previously reported by other authors, which suggests the involvement of molecular mechanisms related to retinoic acid nuclear receptors, RARα and RARβ, to explain this effect [27]. Moreover, other authors have recently described that some retinoids, including ATRA, promote the regeneration of liver mass and function with full recovery after partial hepatectomy [28].

In the light of these data, it seems reasonable to hypothesize that ATRA administration as neoadjuvant therapy could improve the effectiveness of conventional treatment for liver metastasis based on surgery and chemotherapy. Several lines of evidence obtained from different tumor models support this idea; namely, it has been observed that adding ATRA to chemotherapeutic agents, including celecoxib [29], placlitaxel [30], cisplatin [31], and also CY [32], could be an effective treatment for the inhibition of invasion and proliferation of tumor cells. The mechanisms of action of the antitumor effects of these combinations involve apoptosis induction,

by increasing expression of RARβ, and synergistic antiproliferative effect on tumor cells; in addition, in the case of ATRA plus CY, it has been found that this combination creates a favorable host environment by reducing secretion of VEGF and some interleukins, such as interleukin 6 and interleukin 10 [32].

In our model of liver metastases, we also used the prodifferentiation agent ATRA in combination with hepatectomy plus the alkylating agent CY, to determine the value of ATRA as neoadjuvant agent in the treatment of RMS liver metastases. As expected, we observed that adjuvant chemotherapy with CY treatment effectively inhibited the growth of metastases, thereby extending survival of the animals. However, as described previously [33], its use is often accompanied by immunohematological toxicity. In the present study, besides significant weight loss, rats developed anemia, thrombocytopenia, and severe leukopenia, from which there was partial recovery in the third week after starting treatment.

In comparison with the effect of CY therapy, the neoadjuvant treatment based on adding ATRA to CY therapy resulted in greater antitumor activity, significantly increasing not only the median survival, but also the LTS. As well as this therapeutic effect exerted by the ATRA + CY combination, there were some differences in side effects with respect to that induced by CY alone. In particular, although no differences were found in body weight or platelet count, there was a significantly larger decrease in red cell count; in contrast, regarding WBC count, after an initial leukopenia similar to that produced by CY alone, leukocytosis was found in a later phase (third week). Other authors have also described a WBC count increase induced by ATRA and have linked the immunomodulatory and antitumor effects of this retinoic acid in solid tumor-bearing mice [34]. Finally, the ALP levels were also higher when ATRA was combined with CY treatment, this being a characteristic effect of ATRA as described previously.

In summary, we have demonstrated the benefit of ATRA therapy for the treatment of RMS liver metastases. Our results suggest that ATRA overcame the growth-promoting effect of GFs, which could be responsible for the partial block of the stimulating effect of hepatectomy on residual tumor cells. Consequently, the addition of ATRA to CY can produce a significantly greater antitumor activity than obtained with the alkylating agent alone. Taken together, in our view, the neoadjuvant therapy with ATRA could be a promising strategy for improving the results of conventional therapy of liver metastases with no apparent disturbance of liver regeneration.

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REFERENCES

[1] Manfredi S, Lepage C, Hatem C, Coatmeur O, Faivre J, Bouvier A. Epidemiology and management of liver metastases from colorectal cancer. Ann Surg 2006;244:254.

[2] Swan PJ, Welsh FKS, Chandrakumaran K, Rees M. Long-term survival following delayed presentation and resection of colorectal liver metastases. Br J Surg 2011;98:1309.

[3] Chan KM, Chiang J-M, Lee C-F, et al. Outcomes of resection for colorectal cancer hepatic metastases stratified by evolving eras of treatment. World J SurgOncol 2011;9:174. http://dx.doi.org/10.1186/1477-7819-9-174.

[4] De Jong MC, Pulitano C, Ribero D, et al. Rates and patterns of recurrence following curative intent surgery for colorectal liver metastasis: an international multi-institutional analysis of 1669 patients. Ann Surg 2009;250:440.

[5] Stremitzer S, Stift J, Gruenberger B, et al. KRAS status and outcome of liver resection after neoadjuvant chemotherapy including bevacizumab. Br J Surg 2012;99:1575.

[6] Leporrier J, Maurel J, Chiche L, Bara S, Segol P, Launoy G. A population-based study of the incidence, management and prognosis of hepatic metastases from colorectal cancer. Br J Surg 2006;93:465.

[7] García-Alonso I, Palomares T, Alonso A, et al. Effect of hepatic resection on development of liver metastasis. Rev Esp Enferm Dig 2003;95:771.

[8] García-Alonso I, Palomares T, Alonso-Varona A, et al. Effect of liver resection on the progression and growth of rhabdomyosarcoma metastases in a rat model. J Surg Res 2008;148:185.

[9] Christophi C, Harun N, Fifis T. Liver regeneration and tumor stimulation-a review of cytokine and angiogenic factors. J GastrointestSurg 2008;12:966.

[10] Caramés M, Alonso-Varona A, García-Alonso I, Palomares T. Glutathione modulators reverse the pro-tumour effect of growth factors enhancing WiDr cell response to chemotherapeutic agents. Anticancer Res 2010;30:1223.

[11] Palomares T, Caramés M, Garcia-Alonso Montoya I, Alonso Varona A. Growth factors and the redox state as new therapeutic targets for colorectal cancer. In: Ettarh Rajunor, editor. Colorectal cancer biology. From genes to tumor. Croatia: Intech; 2012. p. 189–216.

[12] Chattopadhyay N, Butters RR, Brown EM. Agonists of the retinoic acid- and retinoid X-receptors inhibit hepatocyte growth factor secretion and expression in U87 human astrocytoma cells. Brain Res Mol Brain Res 2001;87:100.

[13] Palomares T, Castro B, Del Olmo M, Iglesias A, Bilbao P, Alonso-Varona A. Influence of the level of gamma-glutamyltranspeptidase activity on the response of poorly and moderately differentiated rhabdomyosarcoma cell lines to all-trans-retinoic acid. Anti-Cancer Drugs 2006;17:1127.

[14] Radominska-Pandya A, Chen G, Czernik PJ, et al. Direct interaction of all-trans-retinoic acid with protein kinase C (PKC). J Biol Chem 2000;275:22324.

[15] García-Alonso I, Palomares T, Alonso-Varona A, et al. Effects of all-trans retinoic acid on tumor recurrence and metastasis. Rev Esp Enferm Dig 2005;97:240.
[16] Barlow JW, Wiley JC, Mous M, et al. Differentiation of rhabdomyosarcoma cell lines using retinoic acid. Pediatr Blood Cancer 2006;47:773.
[17] Gee MF, Tsuchida R, Eichler-Jonsson C, Das B, Baruchel S, Malkin D. Vascular endothelial growth factor acts in an autocrine manner in rhabdomyosarcoma cell lines and can be inhibited with all-trans-retinoic acid. Oncogene 2005;24: 8025.
[18] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official J Eur Union L 2010;276:33.
[19] Bilbao P, del Olmo M, Alonso-Varona A, Castro B, Bilbao J, Palomares T. L-2-Ocxothiazolidine-4-carboxylate reverses the tumour growth-promoting effect of interleukin-2 and improves the anti-tumour efficacy of biochemotherapy in mice bearing B16 melanoma liver metastases. Melanoma Res 2002;12:17.
[20] Harun N, Nikfarjam M, Muralidharan V, Christophi CH. Liver regeneration stimulates tumor metastases. J Surg Res 2007; 138:284.
[21] Lejeune FJ. Is surgical trauma prometastatic? Anticancer Res 2012;32:947.
[22] Suzuki S, Kawakami S, Chansri N, Yamashita F, Hashida M. Inhibition of pulmonary metastasis in mice by all-trans retinoic acid incorporated in cationic liposomes. J Control Release 2006;116:58.
[23] Park EY, Pinali D, Lindley K, Lane MA. Hepatic vitamin A preloading reduces colorectal cancer metastatic multiplicity in a mouse xenograft model. Nutr Cancer(Epub) 2012;64:732.
[24] Andreana L, Burroughs AK. Treatment of early hepatocellular carcinoma: how to predict and prevent recurrence. Dig Liver Dis 2010;42(S3):249.
[25] Lim YC, Kang HJ, Kim YS, Choi EC. All-trans-retinoic acid inhibits growth of head and neck cancer stem cells by suppression of Wnt/β-catenin pathway. Eur J Cancer 2012;48: 3310.
[26] Ning X, Shu J, Du Y, Ben Q, Li Z. Therapeutic strategies targeting cancer stem cells. Cancer Biol Ther 2013;14:295.
[27] Wan Y, Yang S, Sun F, Wang J, Chen Q, Hong A. All-trans retinoic acid induces chromatin remodeling at the promoter of the mouse liver, bone, and kidney alkaline phosphatase gene in C3H10T 1/2 cells. Biochem Genet 2012;50:495.
[28] Kimura M, Watanabe M, Ishibashi N, Yanagida S, Ogihara M. Acyclic retinoid NIK-333 accelerates liver regeneration and lowers serum transaminase activities in 70% partially hepatectomized rats in vivo. Eur J Pharmacol 2010;643:267.
[29] Liu JP, Wei HB, Zheng ZH, Guo WP, Fang JF. Celecoxib increases retinoid sensitivity in human colon cancer cell lines. Cell Mol Biol Lett 2010;15:440.
[30] Hong GY, Jeong YI, Lee SJ, Lee E, Oh JS, Lee H. Combination of paclitaxel- and retinoic acid-incorporated nanoparticles for the treatment of CT-26 colon carcinoma. Arch Pharm Res 2011;34:407.
[31] Arrieta O, Gonzalez-De la Rosa CH, Arechaga-Ocampo E, et al. Randomized phase II trial of all-trans-retinoic acid with chemotherapy based on paclitaxel and cisplatin as first-line treatment in patients with advanced non-small-cell lung cancer. J ClinOncol 2010;28:3463.
[32] Song XX, Guo WZ, Cui JF, et al. A tritherapy combination of a fusion protein vaccine with immune-modulating doses of sequential chemotherapies in an optimized regimen completely eradicates large tumors in mice. Int J Cancer 2011;128:1129.
[33] del Olmo M, Alonso-Varona A, Castro B, Calle Y, Bilbao P, Palomares T. Effects of L-2-oxothiazolidine-4-carboxylate (OTZ) on the cytotoxic activity and toxicity of cyclophosphamide on mice bearing B16F10 melanoma liver metastasis. Melanoma Res 2000;10:103.
[34] Siddikuzzaman, Grace VMB. Evaluation of immunomodulatory and antitumor activity of all trans retinoic acid (ATRA) in solid tumor bearing mice. Immunopharmacol Immunotoxicol 2013;35:110.