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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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ABSTRACT

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.

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).