Effects of all-trans retinoic acid on tumor recurrence and metastasis

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ABSTRACT

Objective: all-trans-retinoic acid (ATRA) promotes cell differentiation. We have studied its effect on the local recurrence and metastatic spreading of an experimental rhabdomyosarcoma in rats.

Design: syngenic rhabdomyosarcoma cells (S4MH) were inoculated s.c. in male WAG/RijCrl rats. After 25 days tumors were excised and a 40% hepatectomy was performed for all animals. Ten days later the rats were sacrificed and a thorough necropsy was performed. The animals were randomly allocated to receive daily doses of ATRA (5 mg/kg, i.p.) or its solvent (Clinoleic®/ethanol 90/10), starting three days before surgery until the end of the experiment.

Results: ATRA reduced the incidence of local recurrence from 70 to 33% ($p < 0.05$), but the tumor size was not altered (1.8 vs. 2.0 cc). Regarding inguinal metastasis, there was a six-fold decrease (0.2 vs. 1.2 cc; $p < 0.05$) in mean tumor volume, although the rate of this proliferation increased sharply (86 vs. 29%; $p < 0.05$) for treated animals. The volume of the retroperitoneal tumor masses also decreased with ATRA (0.7 vs. 5.1 cc; $p < 0.05$), but the difference in rate was not significant (71 vs. 67%). Lung metastases, which were present in 100% of control animals, were found in only 33% of treated rats, while the mean number of metastatic foci dropped from 26.7 to 5.7 ($p < 0.05$).

Conclusion: protocols including retinoid administration prior to and following primary tumor excision could help in controlling both recurrence and metastatic progression in surgically treated rhabdomyosarcoma.

Key words: Rat. Rhabdomyosarcoma. Recurrence. Metastasis. All-trans-retinoic acid.

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INTRODUCTION

Over the past decade, remarkable progress has been made in antineoplastic therapy, with surgery still in the forefront as the main curative approach. However, recurrence of disease following tumor resection remains a major problem, not only because of failed local control but also because of the effect of surgery on the distant proliferation of disease, thus reducing patient quality of life and survival (1).

While tumor recurrence depends to a great extent on the radicality of surgery, it can also be induced by biological responses initiated following tissue aggression as implied by the operation itself. Indeed, in an experimental carcinogenesis model, researchers observed that the rate of colon tumor development following a carcinogen was much higher within healing anastomoses in the colon (2).

Surgery, it must be remembered, triggers local release of multiple growth factors (GF) that are responsible for wound healing, but which may also act as paracrine factors giving tumor cells the signal they require to divide and proliferate (3). Such factors include beta fibroblast proliferation-stimulating GF (FGFb), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and others (4-6). It is important therefore to design combined treatments that will inhibit residual tumor cell proliferation following surgery, without impairment of the healing process.

Today it is well known that a tumor's behavior is related to the degree of differentiation of cancer cells, so that

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usually the lower the degree of differentiation the greater the aggressiveness that may be expected from the neoplasm. Therefore, this study was undertaken to explore a differentiation inducement treatment to be used in combination with surgery as a strategy to prevent the development of both the primary tumor and potential metastases. Retinoic acid and related molecules have been shown to be capable of reducing the aggressiveness of different tumor lines both *in vitro* (7) and *in vivo* (8) by inducing cell differentiation (9) and apoptosis. In this regard, we recently reported that all-trans retinoic acid (ATRA) blocks rhabdomyosarcoma development in male WAG/RijCrl rats inoculated s.c. in the right flank (10).

Using the same experimental model, whose natural development has been reported in detail in earlier works (11), we studied the effects of ATRA on local recurrence following the surgical excision of the tumors induced in the animals' flank. In order to enhance the presence of growth factors locally released in the surgical bed, we associated tumorectomy with partial (40%) hepatectomy, a procedure often used for managing single hepatic metastases in combination with the surgical excision of the primary tumor. Thus, growth factors were also present in the serum of animals, and could then be conveyed to any body site harboring tumor cells.

METHODS

The study was carried out on syngenic, 8-week-old, male WAG/RijCrl rats stabilized throughout the experiment with a 12-h light/darkness cycle and given food (Panlab A-04) and drink *ad libitum*. Spain's National Guidelines for the care of laboratory animals (Royal Decree Law No. 223/88) were followed at all times.

Experimental model

As a tumor model we used the S4MH rhabdomyosarcoma cell line (syngenic for WAG rats), with a high affinity for the development of liver metastasis. Cells (2.5×10^5 cells, resuspended in 250 μ l of Ca^{++} -free PBS [phosphate-buffered saline solution]) were administered by subcutaneous inoculation in the flanks of animals (1 cm ahead of the hip).

Twenty-five days after tumor cell inoculation, the primary malignant lesion was surgically isolated by blunt dissection of the capsular plane, and extirpated along with a 0.5-cm border of skin not attached to the tumor. During the same procedure, a partial (40%) hepatectomy (excision of the right lateral lobe) was performed to simulate a hepatic resection for metastatic disease. To facilitate recovery following surgery, 5 ml of 5% glucose solution were administered i.p. For the next ten days, the animals were monitored for potential local recurrence of the tumor.

The rats were sacrificed on day 35 post-inoculation, and the necroscopic study was performed without the technician knowing which treatment the animals had received. The weight of each animal and liver were recorded, followed by an assessment of local-regional progression, including the measurement of volume and weight for any tumor recurrence and a count of any macroscopical metastases found in inguinal and axillary nodes, as well as in the retroperitoneum. Finally, the number of macroscopical metastases observed on the lung surface was recorded.

All-trans retinoic acid treatment

ATRA (sigma R-2625) was administered i.p. at doses of 5 mg/kg (0.6 ml of [90% clinoleic® + 10% ethanol]). Treatment was begun three days after tumorectomy and then continued until the tenth day post-surgery. To this end, the 14 study rats were randomly separated into two groups, with the control group receiving only the solvent (0.6 ml of [90% Clinoleic® + 10% ethanol]) without ATRA.

To evaluate the possible toxicity of the ATRA treatment, animal weight was recorded over the course of the experiment along with the final liver weight.

Statistical analysis

The statistical analysis was performed by using Fisher's exact test to compare the frequency of glandular metastasis development, and applying Student's t-test for other quantitative parameters.

RESULTS

Effects of ATRA on local tumor progression

Treatment with ATRA was well tolerated by the animals, for while the experimental group lost more body weight than the controls, this loss was always less than 15% of initial body weight (Fig. 1A). Moreover, liver mass, expressed as a percentage of total body weight, did not differ as a result of treatment when compared to the control group (Fig. 1B).

The rate of local recurrence was measured ten days after resection of the primary tumor (day 35 of the neoplastic process). A high rate of local recurrence was registered in the control group, with over 70% of animals affected. In the ATRA treatment group, however, the rate of local recurrence was less than half in the control group (33 vs. 71%, $p < 0.05$) (Fig. 2A). Nevertheless, there were no significant differences between the experimental and control groups in mean volume of recurrent tumor mass (1.8 vs. 2.0 cc, $p = 0.89$) (Fig. 2B).

Fig. 1.- Weights at end of treatment. (A) Mean body weight (g). (B) Mean liver weight (% of body weight).
 Datos ponderales al finalizar el tratamiento. (A) Media del peso corporal (en g). (B) Media del peso hepático (en % sobre peso corporal).

Fig. 2.- Tumor recurrence. (A) Black shows percentage of animals with primary tumor recurrence. (B) Mean volume of local tumor recurrences (cm³).

Recidiva tumoral. (A) En negro se recoge el porcentaje de animales que presentaron recurrencia del tumor primario. (B) Volumen medio de las recurrencias locales del tumor (cm³).

Effects of ATRA on distant tumor progression

To study the dissemination of disease following resection of the primary lesion, as well as the effects of ATRA on tumor progression, an analysis was made of local-regional neoplastic invasion at the inguinal and retroperitoneal levels and of distant invasion by analyzing the rate of pulmonary infiltration and number of foci in the lungs.

With regard to local and regional progression, there was a significantly higher rate of inguinal node invasion in the ATRA-treated animals *versus* the control group (86 vs. 29%; $p < 0.05$). However, the mean volume of in-

Fig. 3.- Inguinal metastases in experimental and control groups. (A) Percentage of animals with ipsilateral inguinal metastasis. (B) Mean volume of metastases detected.

Metástasis inguinales en los dos grupos experimentales. (A) Porcentaje de animales que presentaron metástasis en ganglios inguinales ipsilaterales. (B) Volumen medio de las metástasis encontradas.

guinal metastases in treated animals was 6 times smaller than in the control group (0.2 vs. 1.2 cc; $p < 0.05$).

Moreover, as can be seen in figure 4, there was a high rate of retroperitoneal metastasis in both the control and ATRA therapy groups, with no significant differences between the two (71 vs. 67%, $p > 0.05$). However, the mean volume of retroperitoneal tumor mass was 7 times smaller in the ATRA-treated group than in the control animals (0.7 vs. 5.1 cc; $p < 0.05$).

Concerning distant tumor progression, while in the control group all animals exhibited metastatic invasion of

Fig. 4. Retroperitoneal metastasis. (A) Percentage of animals with retroperitoneal metastasis. (B) Mean volume of metastases detected.

Extensión del tumor al retroperitoneo. (A) Porcentaje de animales que presentaron metástasis retroperitoneales. (B) Volumen medio de las metástasis encontradas.

Fig. 5. Pulmonary metastasis. (A) Percentage of animals with pulmonary metastasis. (B) Mean number of metastases per animal.

Estudio de la afectación pulmonar metastática. (A) Porcentaje de animales que presentaron metástasis. (B) Número medio de focos por animal.

the lungs, in the ATRA-treated group the rate was reduced by 33% ($p < 0.05$) (Fig. 5). Moreover, the count of metastatic foci also showed a 5-fold reduction in the mean number of pulmonary colonies when compared to the control group (5.7 vs. 26.7; $p < 0.05$).

DISCUSSION

Surgery remains the primary treatment for solid tumors. Indeed, their local control depends on appropriate surgical treatment ensuring the removal of the primary lesion together with an ample margin of tumor-free surrounding tissue, and on the biological characteristics of the tumor itself. This is especially true in soft-tissue sarcomas, which still respond poorly to treatment, with little progress having been achieved in recent years (12). In this type of tumor, frequent local invasion greatly complicates surgical excision and gives rise to a high rate of local recurrence (13). Moreover, 40% of soft-tissue sarcoma patients develop distant metastasis, despite apparent local control when the disease is first treated (14). The most important factor for predicting this outcome is histological grade. Indeed, 81% of high-grade sarcomas, such as rhabdomyosarcomas, metastasize within the first year of initial treatment (15). This being the case, tumor type must logically be taken into account in determining the surgical procedure and adjuvant therapy to be followed during the management of the disease.

Furthermore, it should be remembered that the process of invasion and metastasis is influenced by numerous two-way interactions between tumor cells and stromal cells such as fibroblasts, macrophages, lymphocytes and endothelial cells (16,17), and that these interactions are in

turn largely regulated by growth factors (18), whose release is fostered by the surgical act itself.

This means that other approaches in addition to surgery must be developed to reduce the percentage of failed local control through strategies designed to be implemented in combination with the surgical approach, taking into account the actual biology of tumor cells.

In order to experimentally reproduce the clinical problem outlined above, which is characterized by failed local control and the development of metastases following solid tumor surgery, we decided to use as tumor model the S4MH rat rhabdomyosarcoma cell line, because of its high affinity for the development of tumors and metastasis. As a surgical treatment model, we performed a local surgical excision of the primary lesion together with a partial hepatectomy, a combination that prompts the release of a large flow of growth factors into the circulation, thus allowing obtaining a paracrine stimulation to promote invasion and metastasis. Hepatocyte growth factor (HGF), one of the main such factors secreted following hepatectomy, is well known for its role in inducing the growth, motility and invasion of different types of tumor cells (19). To this must be added the tumor-promoting activity of other factors secreted by stromal cells at the surgical wound, such as FGFb, vascular endothelial growth factor (VEGF), TGF- β , interleukins such as IL-6 and IL-8, etc. (20,21).

In designing a treatment to be used in combination with surgery that would take into account the influence of differentiation extent on soft-tissue sarcomas, we decided on all-trans retinoic acid because of its inhibitory effect on tumor cell growth and its ability to stimulate differentiation and apoptosis (22,23). Specifically, ATRA has demonstrated its anti-neoplastic activity on a number of human solid tumors, including mammary carcinomas (24), head and neck tumors (25), ovarian cancer (26), and other malignancies. Moreover, at doses utilized, no adverse side effects worthy of consideration have been described.

It was considered that ATRA therapy should be administered before surgery and continued thereafter to produce differentiation-inductive signaling prior to the release of growth factors due to surgery itself, and also to ensure that the signaling would remain active over a long period of time, so that we could effectively counteract the differentiation-hindering signal –i.e., the cell proliferation stimulus by growth factors.

Our results show that this model results in a high rate of local recurrence and local-regional invasion (characteristic of human soft-tissue tumors), and in the development of metastasis in all animals within the control group. We feel that this confirms the usefulness of our experimental model in assessing the effectiveness of a new anti-neoplastic treatment.

Continuous pre- and post-surgical treatment with ATRA was well tolerated by animals, for although their body weight decreased, the loss amounted to less than 15% of their pre-treatment weight.

Regarding the effectiveness of the treatment strategy tested in this experiment, the addition of ATRA to surgical treatment was associated with a 38% reduction in local recurrence and a 33% drop in the presence of metastasis in the lungs, a preferential target organ for most metastasizing sarcomas (27). This decline in the metastatic capacity of S4MH cells produced by ATRA is reflected not only in the reduced number of animals with metastasis, but also in a drop in the mean number of pulmonary foci, which were only one-fifth the number in the control group. This significant reduction in tumor mass was also observed for retroperitoneal metastases, although their incidence was not reduced.

Regarding node metastases, while their frequency is low (3.9-5.9%) in most sarcomas, in the case of rhabdomyosarcoma it can reach as high as 36% of patients (28). In our experimental model, node invasion was detected in 29% of animals, a percentage that paradoxically increased significantly (86%) in ATRA-receiving rats. However, in this case as well, there was a 6-fold decrease in mean tumor volume in the experimental group when compared to the control group.

In conclusion, this *in vivo* study shows that treatment with ATRA, administered in combination with primary tumor resection, is associated with a reduction in the metastatic potential of rat S4MH rhabdomyosarcoma, suggesting that a therapeutic approach based on pre- and post-extirpation treatment with retinoids could enhance local control for solid tumors subjected to surgery.

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