Growth Inhibition of Cervix Carcinoma Cells in Vivo by Endothelin A Receptor Blockade

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Abstract

In human papillomavirus (HPV)-positive cervical cancer cells, the endothelin A receptor (ET_A) mediates an endothelin-1-induced mitogenic effect, thus representing a relevant target for anti-tumor therapy. Here, we describe the complete inhibition of human cervical carcinoma growth by blocking the ET_A. In nude mice, the ET_A-selective antagonist atrasentan inhibits the growth and the neoangiogenesis of cervical carcinoma xenografts. Two cycles of treatment completely revert tumor growth. Atrasentan displays additive effects when administered in combination with the cytotoxic drug paclitaxel. These results demonstrate that by inhibiting cell proliferation and angiogenesis, this small molecule may help to control cervical cancer by either monotherapy or combination therapy.

Introduction

Cervical cancer is the second most common cancer in women worldwide (breast cancer is the most common) and the leading cause of cancer-related death among women in developing countries. Biologic compounds with low toxicity may help to improve current cervical cancer therapies. The family of ETs, including ET-1, ET-2, and ET-3, are 21-amino acid peptides exerting many biological effects (1). Two major receptor subtypes belonging to the G protein-coupled family receptors mediate ET signals: the ET_A, which binds ET-1 and ET-2 with high affinity and ET-3 with low affinity; and the ET_B, which binds all ET isopeptides with equal affinity (2). ET-1 induces cell proliferation directly or synergistically with other growth factors that are relevant in cancer progression. It has been proposed that ET-1 exerts autocrine or paracrine action on neoplastic and surrounding stromal cells, contributing to development and progression of a variety of malignancies (2, 3). Engagement of ET_A by ET-1 triggers activation of tumor proliferation (3-7), VEGF-induced angiogenesis, (8, 9) invasiveness, (10), and inhibition of paclitaxel-induced apoptosis (11, 12). Human keratinocytes express ET_A and produce ET-1, which sustains an autocrine growth response (13). HPV can immortalize and transform human keratinocytes that secrete ET-1 and exhibit a 2-fold increase in ET_A compared with parental cells in the absence of recycling alteration (14). ET-1 induces an increased growth response in these cell lines (which can proliferate in the absence of any growth factor; Ref. 15). ET-1 and its ET_A are also overexpressed in HPV-associated cervix carcinoma cells (7). Binding studies showed that these transformed cells express an increased number of functional ET_A and that ET-1 (but not ET-3) stimulates a marked dose-dependent increase in [3H]thymidine incorporation compared with the normal counterpart. This proliferative effect is selectively blocked by an ET_A antagonist, whereas an ET_B antagonist has no effect. These results demonstrate that ET-1 is involved in the growth of HPV-associated carcinoma cells overexpressing ET_A and that these receptors are clinically relevant targets for anti-tumor therapy. In this study, we provide evidence that a selective antagonist of the ET_A, ABT-627 (16) is able to inhibit the in vivo growth of HPV-associated cervical carcinoma cells and to potentiate cytotoxic treatment in combination with paclitaxel. This inhibition is associated with a reduced density of tumor-associated microvessels. These results demonstrate the antiangiogenic and anti-tumor effect of this selective ET_A antagonist in cervical carcinoma and support the clinical use of ABT-627 in monotherapy or in combination with cytotoxic drugs.

Materials and Methods

Cell Lines. Cervical carcinoma-derived cell lines, CaSki and C33A, were purchased from American Type Culture Collection. CaSki cells were maintained in RPMI/10% FCS (Invitrogen, Milan, Italy) and C33A cell lines in MEM/10% FCS (Invitrogen). CaSki cells are HPV16-positive, produce ET-1, and express mRNA for ET_A and ET_B. C33A cells, which are HPV negative, do not produce ET-1 and express only mRNA for ET_B (7).

Cell Proliferation Assay. Cells were seeded in 96-well plates at a density (2 × 10^4 cells/well) allowing an exponential growth rate. To induce the inhibition of cell proliferation, paclitaxel (Sigma-Aldrich, Milan, Italy) at 100 μM was added to the cultures for 4 h. ET-1 was added at a concentration of 100 nM. The ET_A antagonist was used at 1 μM by adding the compound 15 min before paclitaxel and/or ET-1. Twenty-four h later, the cellular monolayers were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) for 2 h. The reduced dye was then eluted by isopropanol, and cell metabolic activity was evaluated by measuring the absorbance at A540-620 in a laser microdensitometer. Eight replicas for each point were assayed.

Tumor Cell Xenografts. Female nu/nu mice (Charles River, Cologno Monzese, Italy) were 8 weeks old. Single cell suspension of 1.5 × 10^6 of CaSki and C33A cells with a viability > 95% was injected s.c. into the flank of nude mice. Palpable tumors were detected 7 days after cell injection. Tumor burden was measured with a caliper and calculated as length × width^2 × 0.5.

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Vessel Count. Four weeks after the end of treatment, mice were sacrificed, and tumors were collected. Consecutive 4-µm sections of frozen tissue were stained by H&E or immunostained for CD31 by a rat monoclonal antimonouse CD31 (specific for mouse endothelial cells; a gift from Dr. Alberto Mantovani [Istituto Ricerche Farmacologiche Mario Negri, Milan, Italy]). Immunohistochemical staining was performed by the immunoperoxidase technique (Vector Laboratories, Burlingame, CA). The microvessel number was counted by two different observers in tissue areas, which included higher microvessels density (hot spot).

Statistical Analysis. All experiments were analyzed by the Student’s t test performed by the Instat software (GraphPad Software, Inc., San Diego, CA). All of the Ps resulted from the use of two-sided tests and were considered significant when <0.05.

Results

Growth Inhibition of Cervical Carcinoma Xenograft. Although both cell lines CaSki and C33A were able to produce tumors in nude mice, the CaSki cell tumors had increased tumor mass compared with C33A cells. This difference in tumor burden might reflect the major ability of CaSki cells to proliferate in conditions of low growth factor stimulation (7).

ABT-627 was able to affect the tumor implant at the 2 mg/kg/day dosage, depending on the administration start time. This dosage was chosen because it corresponded to that used in human clinical trials (16). Early treatment (at 0 and 3 days from cell injection) as well as late treatment (at 7 days, when the tumors were already palpable) with the same dosage (2 mg/kg/day) were effective in reducing the size of tumors produced by CaSki cells and in delaying tumor growth (Fig. 1, a–c).

The same dosage of the antagonist ABT-627 that was effective on CaSki xenografts in reducing tumor mass, with maximum activity at early treatment, had no effect on C33A xenografts, as expected because of the absence of ET A R in this cell line (Fig. 1d).

As the single treatment with ABT-627 produced a reduction in tumor mass that was more evident during the treatment and shortly thereafter, a two-cycle treatment was given to the mice. Two cycles of 21-day treatment (with 7-day-intervals) was started when palpable tumors were present in the flank of the mice. This double treatment had a dramatic effect on tumor growth and caused recovery from tumor in 80% of treated mice (Fig. 2). In the remaining animals, the tumor growth inhibition was higher than that produced by a single cycle of treatment. Higher dosages of ABT-627 (10 mg/kg/day) did not increase the reduction of the tumor mass but affected the survival time of the treated animals with a higher survival probability (data not shown).

Blood Vessel Density. ET-1 triggers activation of VEGF-induced angiogenesis by stimulating VEGF production through ET A R-mediated signaling (9). The VEGF-induced angiogenesis may be evaluated by the number of blood vessels within the tumor. ABT-627, at the same dosage affecting tumor growth, produced a statistically significant reduction in the number of blood vessels within the tumor (Fig. 3). In the hot-spot sites of control tumors, 19 ± 3.49 vessels were detected, whereas in ABT-627-treated tumors, only 8 ± 3.44 vessels were present (P = 0.006).

In Vitro Cooperation between ABT-627 and Paclitaxel in Inhibiting Carcinoma Cell Proliferation. We have previously demonstrated that ABT-627 is more effective than BQ 123 in inhibiting proliferation of CaSki cells (17). A dramatic growth inhibition occurred when these cells were treated in vitro with the chemotherapeutic agent paclitaxel, and this effect was counteracted by the presence of ET-1. The metabolic activity of CaSki cells, which was reduced to ~50% by paclitaxel treatment, was increased toward the level of the untreated cells by the presence of ET-1 (Fig. 4A). Treatment with the ET A R antagonist, ABT-627, counteracted the effects of ET-1 and restored tumor sensitivity to paclitaxel. Simultaneous treatment with
ABT-627 and paclitaxel significantly increased the inhibition of cell proliferation by blocking the action of endogenous ET-1 produced by CaSki cells (Fig. 4A).

In Vivo ABT-627 and Paclitaxel Cooperation. CaSki xenografts were highly sensitive to paclitaxel treatment, with a dramatic reduction of tumor mass. Nevertheless, combined treatment with the antagonist ABT-627 was able to induce a better response to the paclitaxel treatment by further reducing the tumor mass by an additional 70% (Fig. 4B).

Discussion
Changes in the homeostasis of growth factor-induced physiological signaling may lead to unbalanced cell growth. Signaling through the ET₆R activates molecular pathways leading to tumor cell proliferation, neovascularization, invasiveness, and protection against apoptosis (4–12). The ET-1 signaling pathway is up-regulated in various human tumors, including ovarian, breast, and prostate cancer (6, 18, 19). The overproduction of ET-1 and the up-regulation of the autocrine loop mediates by ETA R in HPV-associated cervical carcinoma cells indicate that this receptor could be used as a target for therapy. Consequently, this hypothesis, compounds that antagonize the action of ET-1 by blocking ETA R would be able to affect the growth of cervical carcinoma xenografts in nude mice. In this study, we have investigated the action of the ETA R antagonist ABT-627 on the growth of cervical cancer xenografts in monotherapy as well as in association with the chemotherapeutic compound, paclitaxel.

ABT-627 is a nonpeptide-selective antagonist of ETA R that is in use in clinical trials on metastatic adenocarcinoma of the prostate. This compound was more effective than the peptide antagonist, BQ123, in blocking proliferation and growth of HPV-associated cervical cancer cells in vitro (17). As for other growth factor receptor inhibitors, like those for EGFR, we were expecting to find inhibitory effects against the cervical carcinoma xenograft only during treatment, with a substantial tumor regrowth upon the termination of treatment (20). Surprisingly, ABT-627 treatment at a dosage of 2 mg/kg/day for 21 days caused a marked inhibition and subsequent delay of tumor growth for several weeks after treatment in the absence of any toxic effect or weight loss. Early treatment influenced the tumor implant and caused almost complete inhibition of carcinoma cell xenograft growth. The effect of this selective ET₆R inhibitor confirms that in HPV-infected cervical carcinoma cells, the ETA R mediates autocrine tumor growth stimulation. The lack of effect of ABT-627 in C33A xenografts is indicative of its specificity, as it is known that C33A cells do not express the ETA R and are HPV negative. Higher dosages of ABT-627 (10 mg/kg/day) did not increase the reduction of tumor growth (Fig. 4B).
tumor mass, indicating that the lower doses can saturate the receptors on the tumor cell membrane. The action of high doses on the survival time of the treated animals may be explained by other systemic actions of the ET\(_4\)R antagonists on the organism.

ET-1 causes activation of VEGF-induced angiogenesis by stimulating VEGF production (8, 9). This action is mediated by the ET\(_4\)R, and therefore specific antagonists of this receptor may affect the tumor vascularization. Indeed, ABT-627 produced a statistically significant reduction in the number of blood vessels within the tumor, consistent with the similar role of ET-1 in neovascularization of ovarian carcinoma (9).

ABT-627 seems effective in controlling at least two main aspects of tumor growth: proliferation and neovascularization. However, several weeks after the end of the treatment, small tumors were still present. By introducing a second cycle of therapy, the effect on cervical carcinoma xenograft was more pronounced: 80% of treated animals recovered from the tumor and, in the remaining animals, the tumor volume was reduced at low levels with respect to the control. The cured animals remained tumor-free until the end of the observation (12 months). This is the first observation that two cycles of targeted therapy are able to completely revert tumor growth, at least in nude mouse models.

As stated before, ET-1 protects against paclitaxel-induced apoptosis in ovarian tumor cells through its type A receptor (12). In Ca Ski cells, paclitaxel treatment caused an inhibition of cell growth in vitro that was counteracted by the presence of exogenous ET-1. The presence of ABT-627 affects the action of ET-1 and sensitizes the tumor cells to the activity of paclitaxel. In nude mice, the combined treatment was therefore investigated. Ca Ski xenografts were highly sensitive to paclitaxel treatment. Nevertheless, ABT-627 treatment was able to increase the tumor growth inhibition by a single cycle of therapy.

Previous studies by competitive binding data revealed that HPV-positive cervical carcinoma cells predominantly express functional ET\(_4\)R, supporting the hypothesis that the expression and function of ET\(_4\)R in malignant cervical cells is likely to become the predominant form, representing a relevant mechanism in tumor proliferation (7). The present data reinforce this hypothesis and indicate that prolonged treatment with ET\(_4\)R antagonists should be an effective therapy. The ET\(_4\)R antagonist not only inhibits carcinoma cell proliferation but also interferes with neovascularization, presumably by decreasing VEGF production (9). These findings indicate that a single compound may act against several targets of the neoplastic disease, including the potentiation of cytotoxic drug action.

Taken together, our results demonstrate that in vivo, at least in the mouse model, the ET-1/ET\(_4\)R autocrine loop is a target of gene-based therapy and that such therapy is effective alone or in combination with cytotoxic drugs in inhibiting the growth of HPV-associated neoplasia.

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References

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