In vivo Efficacy of Marimastat and Chemoradiation in Head and Neck Cancer Xenografts

Joni B. Skipper^a Lacey R. McNally^b Eben L. Rosenthal^a Wenquan Wang^c
Donald J. Buchsbaum^b

^aDivision of Otolaryngology, Head and Neck Surgery, Department of Surgery, ^bDivision of Radiation Biology, Department of Radiation Oncology, and ^cBiostatistics and Bioinformatics Unit, Medical Statistics Section, Department of Medicine, University of Alabama at Birmingham, Birmingham, Ala., USA

Key Words

Marimastat · Squamous-cell carcinoma, head and neck, mouse · Synthetic matrix metalloprotease inhibitor

Abstract

Objective: To assess the effect of combining a synthetic matrix metalloprotease inhibitor and chemoradiation therapy on tumor growth in a murine model of head and neck squamous-cell carcinoma (SCC). **Methods:** Athymic, nude mice bearing SCC-1 xenografts were used to comprise 4 treatment groups: (1) control receiving vehicle alone, (2) marimastat alone, (3) cisplatin + radiation in combination and (4) marimastat + cisplatin + radiation in combination. The marimastat was administered at a dose of 8.7 mg/kg/day over a 14-day period via a subcutaneous osmotic pump. The control group received vehicle only via a subcutaneous osmotic pump. Radiotherapy was given in 4 fractions of 8 Gy divided over days 8, 12, 16 and 20 with 4 intraperitoneal doses of cisplatin (3 mg/kg) 1 h before each fraction of radiation. **Results:** Animals receiving triple treatment had delayed growth, measured as lengthened tumor doubling time, compared to the cisplatin + radiation combination ($p = 0.03$). Also, compared to control, the triple-treatment group ($p = 0.005$) had delayed growth in terms of doubling time. Factor VIII immunohistochemistry to assess microvessel density did not dem-

onstrate a reduction in neovascularization between the triple-treatment and cisplatin + radiation combination groups. Statistical analysis failed to demonstrate any significant difference among groups. **Conclusions:** Chemoradiation + marimastat therapy had delayed tumor growth, compared to the chemoradiation alone. Based on these results, marimastat may work in combination with chemotherapy and radiation to inhibit tumor growth. Copyright © 2008 S. Karger AG, Basel

Introduction

The matrix metalloproteases (MMPs) are a family of zinc-dependent proteases involved in the degradation of extracellular matrix components. These proteins have been found to be frequently overexpressed in malignant tumors and have been associated with both an aggressive malignant phenotype and an adverse prognosis in cancer patients [1–4]. Tissue inhibitors of matrix metalloproteases are endogenous inhibitors of MMPs that are thought to balance the matrix remodeling activities of the MMPs [5–8]. The search for advances in cancer treatment has led to the development of matrix metalloprotease inhibitors (MMPIs) [9]. Synthetic compounds were designed to inhibit the tumorigenic potential of MMPs elaborated by

tumor cells, which have been found to be secreted from both tumor cells and surrounding fibroblasts [10, 11]. However, clinical trials involving marimastat, an oral MMPi, alone have been disappointing as many patients failed to benefit from the drug and also experienced significant side effects, such as musculoskeletal toxicity [12]. Although a phase II randomized study in esophageal adenocarcinoma has evaluated the use of MMPs with chemotherapy and radiation [13], no conclusions about the activity of MMPi could be drawn due to the small number of patients enrolled. There is data to indicate that radiotherapy promotes MMP-dependent growth and invasion [14]. Although there are reports published using MMPi in combination with radiation with success [15, 16], the addition of chemotherapy to this regimen has not been reported with squamous-cell carcinoma (SCC).

The phenomenon of radiosensitization has been identified as a mechanism of enhanced tumor response to cisplatin-based therapy and has accounted for significant success rates of combination chemotherapeutic agents and radiation in the last decade [17]. The effectiveness of radiation therapy to combat primary tumors is improved in animal models by the addition of antiangiogenic agents [18–22]. Targeted therapies have also been identified as radiosensitizers [23]. Based on this premise, the purpose of this study was to evaluate the effectiveness of an MMPi, marimastat, when used in conjunction with chemoradiation.

Materials and Methods

Cell Lines and Reagents

A human head and neck cell line, SCC-1, was obtained from the American Type Culture Collection (Manassas, Va., USA). SCC-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with L-glutamine and 10% fetal bovine serum. For xenografts, cells were initially injected into athymic, nude mice (NCI-Frederick, Frederick, Md., USA) to grow for future xenografts into mice for in vivo studies. Cisplatin was purchased from Sigma as a stock solution of 1 mg/ml. For animal studies, cisplatin was diluted in 0.9% saline immediately before injection. Alzet osmotic pumps (Durect Corp., Cupertino, Calif., USA) were filled with marimastat (British Biotech, Oxford, UK) prior to subcutaneous implantation.

Animal Models

Three-month-old female nude mice were inoculated using a trochar needle with 2 mm² established SCC-1 tissue subcutaneously in the flank. Treatment started once the tumors were 5–6 mm in diameter. Mice were randomly divided into groups of 8 mice to receive different treatments: (1) control, (2) marimastat alone, (3) cisplatin + radiation in combination and (4) marimastat + cisplatin + radiation in combination. All animals received a 14-

day osmotic pump containing dimethylsulfoxide (DMSO) as a control for both the pump and vehicle. Animals treated with marimastat received the same osmotic pump containing 200 µl of marimastat with DMSO to result in a daily dose of 8.7 mg/kg 10 days after the initiation of treatment. Lead-shielded animals received 8 Gy of ⁶⁰Co radiation to the exposed tumor, divided into 4 fractions on days 8, 12, 16 and 20. A dose of 8 Gy was chosen because 7.5 Gy (7,500 rad) had been shown in previous experiments to inhibit tumor growth without being a curative dose [24]. Animals received 4 intraperitoneal doses of cisplatin (3 mg/kg) 1 h before each fraction of radiation. Tumors were measured bi-weekly for 32 days. Potential treatment toxicity was monitored using mouse weight. Tumor size (surface area equal to product of two largest diameters) and regression rates were determined in each treatment group. After 32 days, tumors were harvested for immunohistochemistry. Day 32 was chosen due to death of control group animals and euthanization of animals showing clinical signs of illness to allow for statistical analysis of data acquired from surviving animals.

Immunohistochemistry for Angiogenesis

Tissue was removed from treated xenografts at the end of the study and fixed into paraffin blocks. Angiogenesis was determined using a factor VIII antibody (Sigma-Aldrich, St. Louis, Mo., USA). Formalin-fixed, paraffin-embedded tumors were cut into 4-µm sections and put onto slides. After these had been deparaffinized and rehydrated, antigen retrieval was performed in a pressure cooker for 20 min in pH 8 EDTA buffer. After H₂O₂ quench and 3% horse serum blocking, slides were incubated with the primary antibody at 1:1,000 dilution for 20 min followed by Signet horseradish peroxidase label for 20 min and Biogenex diaminobenzidine for 7 min. Slides were counterstained with hematoxylin. Hematoxylin-eosin-safranin staining was performed on all of the xenografts for morphology. Angiogenesis scores were determined using a grid method, and statistical differences were determined using ANOVA.

Statistical Analysis

Tumor area data for each animal were transformed to represent percent change from baseline, i.e. (tumor area – tumor area at 8 days)/tumor area at 8 days · 100%. A polynomial linear regression, including days after baseline and square term of days after baseline, was fitted for each animal's tumor growth. Time to tumor doubling for each animal was calculated based on the regression model. Area under the growth curve (AUC) for each animal was also calculated. To account for the heterogeneous follow-up time, which was dependent on the tumor growth, AUC was divided by the total days of follow-up (averaged AUC), i.e. from baseline 8 days to the end of study or animal death. Since a few animals did not have tumor doubling, the log rank test was also applied to compare time to tumor doubling between groups.

Results

Athymic, female nude mice were randomly divided into 4 treatment groups (n = 8 per group) and underwent subcutaneous flank trochar implantations of SCC-1 tu-

Table 1. The 4 treatment groups

Group	Cisplatin ¹	Radiation ²	Marimastat	DMSO
Control				x
Marimastat			x	
Chemoradiation	x	x		
Triple treatment	x	x	x	

Each group was comprised of 8 mice to allow for statistical calculation. The control group received DMSO only via a subcutaneous osmotic pump. The marimastat group also utilized osmotic pumps for delivery of the agent. The chemoradiation group received a combination of cisplatin and radiation, whereas the triple-treatment group received all 3 treatment modalities.

¹ 3 mg/kg on days 8, 12, 16 and 20.

² 2 Gy on days 8, 12, 16 and 20.

mor harvested from existing tumors in athymic mice. Measurement of tumor growth began once the tumors had reached an average area of 30 mm², which coincided with day 8 after inoculation. The treatment regimen was delivered over the course of 3 weeks (table 1).

Animals receiving chemoradiation + marimastat had statistically significant delayed growth, compared to animals receiving chemoradiation alone ($p = 0.0299$; fig. 1). Compared to controls, the chemoradiation + marimastat group ($p = 0.005$) had statistically significant delayed growth. Of note, the only animal that demonstrated tumor regression was in the triple-treatment group. However, the MANOVA test failed to demonstrate any statistical difference among all groups.

Factor VIII immunohistochemistry was performed on tumors harvested 30 weeks after treatment (fig. 2). There was no difference noted in microvessel density between the chemoradiation + marimastat group compared to the chemoradiation-alone group ($p = 0.2956$; fig. 3).

Discussion

Combination therapy with differing mechanisms of action is a mainstay of oncology treatment, because it affords several important advantages not achievable with single-agent treatment. Combination therapy is widely used because (1) maximal cell kill within the range of toxicity tolerated by the patient for each agent is achieved, (2) a broader range of interactions occurs (in a heterogeneous tumor population) and (3) there is a potential for

Fig. 1. Tumor growth. The first time point represents the original size of the tumor, and the graph represents the average percent growth from that initial size as the study progressed and treatment ensued. The control group exhibited the highest growth, the triple-treatment group showed the least growth, and the chemoradiation-alone and marimastat-only groups were intermediate in growth by comparison.

preventing or slowing the development of treatment resistance [25].

The use of a cytostatic agent in combination with the cytotoxic radiation and chemotherapeutic regimens has been supported by numerous reports of an enhanced tumor-killing response with the addition of the cytostatic agent [26]. Generally, these studies have sought to improve the killing response to radiation of radiation-sensitive or radiation intermediate cell line xenografts. SCC-1 has previously been shown to be resistant to radiation and to some cytostatic agents in vivo as well as in vitro [27]. Because SCC-1 cells are resistant to radiation therapy alone, the tumors receiving radiation alone continued to grow throughout treatment (table 1). Qian et al. [15] found that irradiation significantly inhibited cell proliferation and migration in some pancreatic cells; however, irradiation also enhanced the invasive potential in some of those cells. This biological effect of irradiation involved enhanced MMP-2 activity in vitro, a previously unknown mechanism. When the MMPI CGS27023A was administered simultaneously, it suppressed the radiation-enhanced invasion by preventing the transition of MMP-2

Fig. 2. To assess differences in neovascularization, factor VIII staining was performed on the chemoradiation + marimastat, the chemoradiation-alone group and the control group receiving DMSO only. The triple-treatment group did not show a reduction in neovascularization compared to the chemoradiation group or the control group.

Fig. 3. Quantification of angiogenesis in SCC-1 xenografts. Tumors were excised from nude mice 14 days after treatment. Treatment of tumors was as follows: (1) marimastat + radiation + cisplatin (MRC); (2) control; (3) radiation + cisplatin (RC). Angiogenesis was determined using a factor VIII antibody. Blood vessels were counted in 3 images per treatment using a standard grid for quantification. No significant differences were found ($p = 0.2956$).

from the latent to the active type [15]. In another study by Kaliski et al. [16], ionizing radiation was found to induce vascular endothelial growth factor secretion by B16 melanoma cells. Metastat, an MMPI, inhibited this secretion of vascular endothelial growth factor. In addition, partial inhibition of ionizing-radiation-induced melanoma cell invasiveness was demonstrated by an anti-vascular-endothelial-growth-factor monoclonal antibody [16]. Angiogenesis, as measured by factor VIII immunohistochemistry (fig. 2, 3), was not statistically different between the control group and the marimastat + radiation + cisplatin treatment.

The single-modality therapy using MMPIs has not been found to be effective in clinical trials [27]. Although the phase II trial for esophageal adenocarcinoma demon-

strated equivocal results [13], treatment with MMPI and chemoradiation in SCC remains untested. In an effort to evaluate previous studies involving the use of an MMPI as a treatment modality, both alone and in combination with radiotherapy, we used marimastat in combination with chemotherapy and radiation because the use of combined platinum-based therapies and radiation are becoming the standard of care. The rationale for the addition of cisplatin stems from the radiosensitization observed when combination chemotherapy and radiation are administered, and the antiangiogenic properties of the MMPI would be expected to contribute to radiosensitization in this model. Our results from this murine model of head and neck tumor xenografts undergoing the described treatment modalities support this hypothesis.

Conclusion

Chemoradiotherapy in combination with marimastat had delayed tumor growth compared to the cisplatin and radiation combination group. Chemoradiotherapy in combination with marimastat did not alter tumor angiogenesis compared to the control group. Based on these results, marimastat may have an effect as a radiosensitizer.

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