Roxithromycin Inhibits Angiogenesis of Human Hepatoma Cells In Vivo by Suppressing VEGF Production

DAI AOKI1, SHINICHI UENO1, FUMITAKE KUBO1, TOHRU OYAMA2, TETSUYA SAKUTA2, KENJI MATSUSHITA2, IKURO MARUYAMA3 and TAKASHI AIKOU1

1Department of Surgical Oncology and Digestive Surgery, 2Department of Operative Dentistry and Endodontology and 3Department of Laboratory and Molecular Medicine, Kagoshima University, School of Medicine, Kagoshima, Japan

Abstract. Background: Recently, 14-member macrolide antibiotics such as Clarithromycin and Roxithromycin (RXM) have been shown to have anti-cancer and anti-angiogenic effects. However, it is not fully understood whether and how RXM suppresses angiogenesis in human hepatoma, which is a well-known hypervascular tumor. Materials and Methods: In the present study, we examined the effects of RXM on tumor angiogenesis in the human hepatoma cell line, HepG2. In vivo, angiogenesis was examined using a mouse dorsal air sac model. Results: The inhibitory effect of RXM was dose-dependent and the angiogenesis index of 100mg/kg/day of RXM administered intraperitoneally twice a day was significantly lower than the control. Next, we examined the effect of RXM on vascular endothelial growth factor (VEGF) mRNA expression and its protein level in HepG2 cells. When 100 μM of RXM were added, VEGF mRNA expression in HepG cells was inhibited and its protein level reduced. Conclusion: These results suggest that RXM inhibits tumor angiogenesis in human hepatoma, and that VEGF alteration may be involved in the mechanism of this inhibitory effect. Because RXM is widely used in clinical practice, it may represent an effective new strategy for human hepatoma therapy.

Angiogenesis is not only involved in tumor growth and distant metastasis, but is also an important early step in carcinogenesis. It is a complex multistep process regulated by a number of angiogenic factors (1-5). Consequently, inhibition of angiogenesis may lead to control of tumor growth and metastasis. It was reported that tumor growth is angiogenesis-dependent by the discovery of angiostatin or endostatin, which specifically inhibit endothelial proliferation (6,7). Now, there are several drugs which have an effect as angiogenesis inhibitors, however most of those are restricted by excessive toxicity and limited efficacy.

Roxithromycin (RXM), which is a new 14- member macrolide antibiotic, has a wide antibacterial spectrum against pathogens and an immunomodulatory effect. Recently, it was shown that 14-member macrolides, which have been reported to be effective in the treatment of chronic lower respiratory tract disease, may act as anti-inflammatory rather than as anti-bacterial agents (8-10). Interestingly, regarding tumor angiogenesis, Yatsunami et al. (11) reported that RXM at concentrations greater than 20 μM inhibited endothelial cell migration and tube formation. Moreover, we reported the strong inhibitory effect of RXM on tumor necrosis factor-alpha (TNF-α) -induced vascular endothelial growth factor (VEGF) expression in human periodontal ligament cells in culture (12). In the mechanism, RXM suppressed activation of the transcription factors AP-1 and SP-1, which were critical factors in VEGF transcription. VEGF is produced constitutively by several cancer cells and is known to be a strong mediator of angiogenesis in a typical hypervascular tumor, human hepatoma (13). We hypothesized that RXM might inhibit angiogenesis through inhibition of VEGF production from human hepatoma cells. Its actions against angiogenesis of HepG2 were studied using an air sac assay model and compared with TNP-470, a well-known angiogenic inhibitor.

Materials and Methods

Correspondence to: Shinichi Ueno, Department of Surgical Oncology and Digestive Surgery, Field of Oncology, Course of Advanced Therapeutics, Kagoshima University Graduate School of Medicine and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890, Japan. Tel: 81-99-275-5361, Fax: 81-99-265-7426, e-mail: ueno1@m.kufm.kagoshima-u.ac.jp

Key Words: Roxithromycin (RXM), VEGF, angiogenesis, HepG2.
Mouse dorsal air sac model of angiogenesis. To examine the effect of RXM on tumor-associated angiogenesis, we used the mouse air sac assay model (11). HepG2 cells were washed twice with PBS and suspended in PBS at 1.34x10^7 cells/ml. A Millipore chamber (diameter, 10mm; thickness, 2 mm; filter pore size, 0.22 µm; Millipore Co., Billerica, MA, USA) was filled with 150 µm (2.0x10^5 cells) of either cell suspension or PBS and implanted subcutaneously into an air sac formed previously in the dorsum of 7- to 8 - week-old male BALB/c mice by the injection of an appropriate volume of air. RXM (40 and 100 mg/kg/day) and TNP-470 (50 mg/kg/day) were administered i.p. every 12 h for 5 days. Six mice in each group were killed and carefully skinned on the fifth day after implantation. The implanted chambers were removed from the subcutaneous air fascia of the treated animals. Angiogenic response was assessed under a dissecting microscope by determining the number of newly formed blood vessels longer than 3 mm with the characteristic zigzagging pattern of tumor cell-induced new vasculature in the subcutaneous side on the skin area that had been in contact with the chamber. The angiogenic response was graded as 0, 1, 2, 3, 4, or 5 according to the number of newly formed blood vessels.

Effect of RXM on HepG2 cell growth. A cell proliferation assay was performed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HepG2 cells were seeded on a 96-well plate in M199 medium containing 10% FBS, as described above. The cells were treated with various concentrations (1-1000 µM) of RXM for 48 h. The number of cells was determined by the MTT assay. Each value is the mean of quadruplicate analysis.

Measurement of VEGF in the culture supernatant of HepG2 cells. The cells (1x10^5 /ml) were seeded on a 90-mm^2 plate in the medium with 1% FBS and cultured with various concentrations of RXM for 48 h. After centrifugation, the supernatant fluid was stored at -70ºC before the assay. VEGF concentrations were determined with enzyme-linked immunosorbant assay kits (Amersham International, Buckinghamshire, UK).

Effect of RXM on VEGF-mRNA expression in HepG2 cells. The cells (1x10^6 /ml) were seeded on a 90-mm^2 plate in the medium with 1% FBS and cultured with various concentrations of RXM for 48 h. Total RNA was isolated from the sediments by the guanidinium-phenol-chloroform (AGPC) method. Isolated RNA was fractionated in 1.2% agarose gel containing 0.66M formaldehyde and transferred to a nylon membrane (Zeta-Probe; BioRad Laboratories, Hercules, CA, USA) by electroblotting. The RNA was cross-linked to the filter with UV-linker (Funa-UV-Linker; Funakoshi Co., Tokyo, Japan). The membrane was prehybridized overnight at 42 ºC with 50% formamide, 1% SDS-4×SSPE (1×SSPE; 180mM NaCl, 10mM NaHPO₄•H₂O, and 1mM EDTA), 0.5% skim milk and 0.5mg of salmon DNA per milliliter. Human VEGF cDNA probe was labeled with [α-32P] deoxy-CTP using a Random Primed Labeling Kit (Boehringer Mannheim, Mannheim, Germany) and added to the prehybridization solution at approximately 10^9 cpm per milliliter. After hybridization for 8 h at 42 ºC, the membrane was washed and exposed to the imaging plate for analysis using a Bioimaging Analyzer (BAS 1000 Mac; Fuji Photo Film Co., Tokyo, Japan).

Statistical analysis. The data were analyzed for significance by Student’s t-test (two-tailed). A p-value of less than 0.05 was considered statistically significant.

Results

Mouse dorsal air sac model of angiogenesis. The chamber containing HepG2 cells induced marked angiogenesis on the fifth day after implantation; however, RXM administered i.p. twice daily inhibited the induction of angiogenesis by HepG2 cells (Figure 1). The inhibitory effects of RXM were dose-dependent and the angiogenesis index (±SE) of the control (5.2±0.98) was significantly higher than the other groups (treatment with 40, 100mg/kg/day of RXM, and 50mg/kg/day of TNP-470 = 3.3±0.82; 3.0±0.89; 2.2±1.17) (Figure 2). Treatment with TNP-470 inhibited tumor-induced angiogenesis more strongly than the treatment with RXM. Implantation of the chamber containing PBS only did not cause any angiogenesis, indicating that this mechanical maneuver itself did not cause angiogenesis. The mice looked healthy during the experiments and did not show any sign of toxic effects or body weight reduction during treatment with RXM. Thus, RXM administered systemically is a potent inhibitor of tumor-induced angiogenesis in vivo.

Effect of RXM on HepG2 cell growth. RXM at concentrations up to 100 µM did not inhibit the growth of HepG2 cells by the MTT assay (Figure 3). However, treatment with RXM at concentrations greater than 100 µM for 2 days reduced the cell growth of HepG2 cells, indicating that these concentrations of RXM were equally toxic to HepG2 cells and the inhibition of in vivo angiogenesis by RXM.

Effect of RXM on VEGF-mRNA expression in HepG2 cells. We examined the effect of RXM on VEGF mRNA expression in HepG2 cells. When 50 µM of RXM were added, VEGF mRNA expression was clearly inhibited (Figure 4). These data indicate that inhibition of in vivo angiogenesis by RXM administered systemically may be attributable to its effects on the implanted HepG2 cells.

Measurement of VEGF in the culture supernatant of HepG2 cells. We measured the VEGF concentration in the culture supernatants of HepG2 cells treated with RXM. Without RXM treatment, HepG2 cells (1x10^5/ml) produced 15.0±0.2 ng/ml of VEGF over 48 h (Figure 5). Treatment with 100 µM RXM (a maximum safety dose) reduced HepG2 cell production of VEGF.
Discussion

The present study on the mouse dorsal air sac model of angiogenesis has revealed that RXM, a 14-member macrolide antibiotic, inhibits the number of newly developed neovasculatures in HepG2 cells in a dose-dependent manner. Furthermore, it has been shown that RXM reduces the production of VEGF in HepG2 cells in vitro at the mRNA and protein levels.

It has been suggested that macrolide antibiotics exert anti-cancer effects by activating macrophages and NK cells through the production of IL-4 (14), or inducing the production of peripheral IL-12 (15), whereas Clarithromycin and Erythromycin have been reported to reduce neutrophil production of IL-8, which may facilitate angiogenesis (16). The present study revealed that one of the anti-cancer effects of RXM against HepG2 cells comes from the anti-angiogenic effect through the inhibition of VEGF actions. Yatsunami et al. (11) reported that treatment with RXM for 24 hours at the highest dose of 50 μM did not inhibit the production of IL-8 and VEGF in H157 cells, which were derived from lung cancer cells, but inhibited endothelial cell migration and tube-like formation. However, our past studies on HPDL cells (12) and the present study on HepG2 cells have revealed that 10 or 100 μM of RXM inhibited VEGF actions directly at the mRNA level or at the protein level, emphasizing the need for future study that would examine the cell type specificity and dose-dependency of these effects. Our present study also showed that treatment with 100 mg/kg of RXM exerted effects not more than that of 50 mg/kg TNP-470, which is a well-known anti-angiogenic drug, consistent with the results provided by Yatsunami et al. (11,17)

As shown in Table I, it has been reported that RXM has several cancer effects and Clarithromycin, which is also a 14-member anti-macrolide antibiotic, has already been used as an activator of immunological competence or as a modulator of
Figure 2. Inhibitory effect of RXM and TNP-470 on the angiogenic response. Five days after the implantation of a chamber containing HepG2 cells, the effect of RXM and TNP-470 on angiogenesis was assessed. Statistical significance was determined by Student’s t test (mean±SE; n=6). *, p=0.0039, **, p=0.001, ***, p<0.001.

Figure 3. Effects of RXM on HepG2 cell growth. HepG2 cells were treated with various concentrations of RXM. The number of the cells was determined by MTT assay.
chemotherapy in clinical medicine. RXM has been used worldwide as a p.o. drug for many years. However, the dose of RXM used in the angiogenesis experiments was more than 10 times higher than the dose of the drug in clinical use. Thus, in the therapy of human hepatoma, RXM derivatives is a potential method, because transarterial chemoembolization therapy using lipiodol is often applied. The collaborative study with the Esai pharmaceutical company has shown that the solubility of RXM in lipiodol is excellent and the stability of RXM in lipiodol for 24 hours at 37°C is approximately 95% (data not shown).

In conclusion, RXM might be considered as a potential anti-cancer therapy for future clinical development.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 14370559 to Shinichi Ueno, Ikuro Maruyama and Takashi Aikou).

References

1 Folkman J: What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst 82: 4-6, 1990.

Figure 4. Effect of RXM on VEGF mRNA expression in HepG2 cells. HepG2 cells were treated with 1, 10 and 50 μM of RXM in 1% FBS containing M199 for 48 h. Northern blot analysis was performed with VEGF and GAPDH cDNA probe. As positive control, the RNA from HepG2 cells -treated medium alone.

Figure 5. Effect of RXM on VEGF production by HepG2 cells. HepG2 cells were treated with 1, 10 and 50 μM of RXM in 1% FBS containing M199 for 24, 48, and 72 hours.
<table>
<thead>
<tr>
<th>Author (Year)</th>
<th>Agent</th>
<th>Effect</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yatsunami11 (1997, 98)</td>
<td>CAM, RXM</td>
<td>Anti-angiogenesis and migration</td>
<td>Inhibition of EC tube formation (lung cancer cells)</td>
</tr>
<tr>
<td>Sakamot18 (1998)</td>
<td>CAM</td>
<td>Improvement of host factor</td>
<td>Enhanced NK cell activity</td>
</tr>
<tr>
<td>Yatsunami17 (1999)</td>
<td>CAM, RXM</td>
<td>Anti-tumor growth and metastasis (melanoma cells)</td>
<td>Anti-angiogenesis as shown above</td>
</tr>
<tr>
<td>Ours (2004)</td>
<td>RXM</td>
<td>Anti-angiogenesis (Hepatoma cells)</td>
<td>Inhibition of VEGF production</td>
</tr>
</tbody>
</table>

Note: CAM, Clarithromycin; RXM, Roxithromycin; EC, endothelial cell; NK, natural killer.

---