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Anticancer effect of rapamycin on MCF-7 via downregulation of VEGF expression

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Abstract The importance of mTOR signaling in tumor biology is widely accepted and a number of agents that selectively target mTOR are being developed in cancer therapy. On the other hand, it has been demonstrated that mTOR can act as an angiogenic agent. Thus, we hypothesized that the mTOR inhibitor-induced anticancer effect is affected by expression of a key angiogenic factor, vascular endothelial growth factor (VEGF) and investigated the anticancer effect underlying mTOR using an in vitro assay. The mTOR inhibitor rapamycin dose-dependently reduced the cell viability of the breast cancer cell line, MCF-7, but did not reduce the cell viability of the colon cancer cell line, HT-29. Rapamycin reduced the VEGF expression in the culture medium of MCF-7, while rapamycin did not contribute VEGF expression in the culture medium of HT-29. VEGF stimulated cell viability and VEGF inhibition reduced cell viability of MCF-7, and rapamycin dose-dependently restored the cell viability of MCF-7 reduced by rapamycin. These findings suggest that mTOR acts as a direct anticancer agent and that the mTOR-inhibitor-induced anticancer effect involved the reduced expression of VEGF in MCF-7. Our results imply that mTOR regulates the expression of VEGF and is involved in breast cancer progression.

Keywords Rapamycin · VEGF · mTOR · MCF-7

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Introduction

The mammalian target of rapamycin (mTOR) is a member of a family of high-molecular-mass protein serine/threonine kinases involved in cancer development, progression, and resistance to antineoplastic agents (Bjornsti and Houghton 2004; Boulay *et al.* 2005; Liu *et al.* 2005; Bufalo *et al.* 2006; Borders *et al.* 2010). Thus, mTOR is a target for anticancer agents; indeed, a new class of anticancer agents, mTOR inhibitors, has recently been developed. Preclinical and clinical evidence shows that everolimus, a rapamycin derivative, has direct anticancer effects, and that mTOR inhibition can enhance the efficacy of endocrine therapy in breast cancer (Boulay *et al.* 2005; Baselga *et al.* 2009; Bachelot *et al.* 2012; Yardley *et al.* 2013). However, the mechanism of rapamycin's action as an anticancer agent is not fully understood.

Recent studies have indicated that mTOR plays a pivotal role in angiogenesis and that rapamycin is an antiangiogenic factor (Guba *et al.* 2002; Tsutsumi *et al.* 2004; Bufalo *et al.* 2006). The antiangiogenic effect of rapamycin was suggested to involve the reduced expression of vascular endothelial growth factor (VEGF). VEGF is a major potent positive regulator of angiogenesis, the effects of which on cancer growth and development have been well documented (Folkman 1995; Bachelder *et al.* 2001; Mercurio *et al.* 2005; Lee *et al.* 2007; Barr *et al.* 2008; Perrot-Applanat and Benedetto 2012). In breast cancer, VEGF has the ability to promote the growth of cancer and act as a survival factor for breast cancer cells (Bachelder *et al.* 2001; Mercurio *et al.* 2005; Lee *et al.* 2007; Barr *et al.* 2008). However, information regarding the role of VEGF in rapamycin's anticancer effect is sparse at present.

In this study, we investigated the anticancer effect underlying mTOR using an in vitro assay. We here demonstrate the effect of rapamycin on MCF-7, a breast cancer cell line, via its

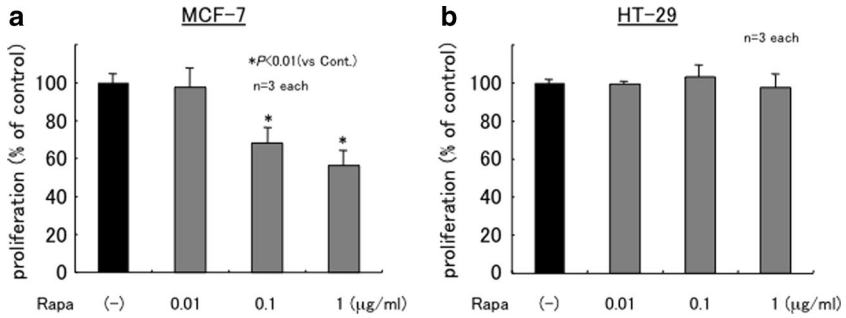

Figure 1. Rapamycin dose-dependently reduced cell viability of MCF-7, but did not reduce cell viability of HT-29. Twenty-four hours after preincubation under the serum-free conditions, MCF-7 (*a*) and HT-29 (*b*) were treated with or without rapamycin (100 ng/ml), an mTOR inhibitor. Twenty-four hours later, cell viability was determined via MTT assay. *$P<0.05$.

regulation of VEGF expression. Our results imply that rapamycin contributes directly to an anticancer effect, at least in part, by regulating VEGF expression.

Materials and Methods

Cells and regents. Human breast cancer cell line (MCF-7) and human colon cancer cell line (HT-29) were from the American Type Culture Collection. The cells were cultured in an RPMI1640 medium (Wako, Japan) supplemented with 10% fetal bovine serum. Rapamycin (Sigma-Aldrich, Tokyo, Japan) was used at the concentration of 100 ng/ml, as previously described (Tsutsumi *et al.* 2004; Fujii *et al.* 2006; Fujii *et al.* 2008). The neutralizing antibody (anti-VEGF from rabbit) was from NeoMarkers Co., Ltd. (Fremont, CA). Cell viability was determined via MTT assay. Cells were seeded in 96-well flat-bottom microtiter plates at a density of 1×10^4 cells, 100 μl per well. After treatment as shown in the figure legends, 10 μl of the cell counting solution (WST-8, Dojindo Laboratories, Tokyo, Japan) was added to each well and the culture was incubated in a humidified 5% CO_2 atm at 37°C for 3 h. Crystals were dissolved in 100 μl of 1 N HCl/well. The absorbance of the solution was read at 650 nm using a microtiter plate reader (Becton Dickinson). Cell viability was calculated according to the following formula: cell viability (%)= A450/A450 (control group)×100.

Enzyme-linked immunosorbent assay. The time course of endogenous VEGF protein contents in culture medium was determined using Quantikine Immunoassay systems for human VEGF-A (R&D Systems Inc., Minneapolis, MN).

Statistical analysis. All data are expressed as means±SD and were analyzed by one-way ANOVA with Fisher's adjustment. $P<0.05$ was considered to be statistically significant.

Results

To assess the direct effect of rapamycin, we examined the cell viability and the induction of VEGF expression using cultured human cells, MCF-7 and HT-29. As shown in Fig. 1, rapamycin dose-dependently reduced the cell viability of MCF-7, but did not reduce the cell viability of HT-29. As shown in Fig. 2, rapamycin reduced the VEGF expression in

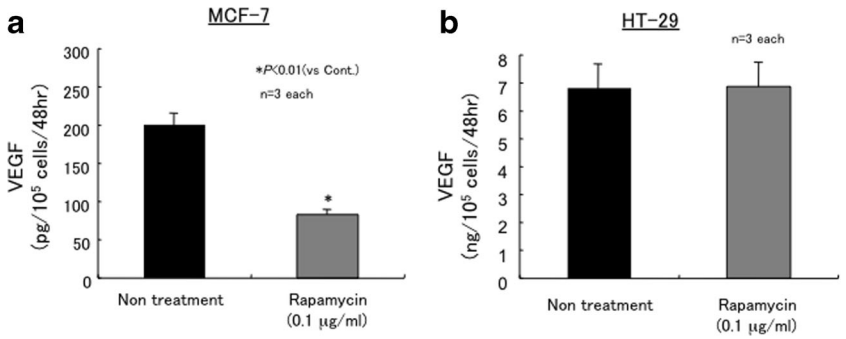

Figure 2. Rapamycin reduced the VEGF expression in the culture medium of MCF-7, while rapamycin did not contribute to the reduction of VEGF expression in the culture medium of HT-29. Twenty-four hours after preincubation under the serum-free conditions, MCF-7 (*a*) and HT-29 (*b*) were treated with or without rapamycin (100 ng/ml). Forty-eight hours later, the culture medium was subjected to ELISA of VEGF. *$P<0.05$.

the culture medium of MCF-7, while rapamycin did not contribute to the reduction of VEGF expression in the culture medium of HT-29. These findings suggest that rapamycin has a direct effect on MCF-7 but does not show a direct effect on HT-29.

As shown in Fig. 3, VEGF dose-dependently stimulated the cell viability of MCF-7, and anti-VEGF neutralizing antibody significantly reduced the cell viability of MCF-7. In contrast, as shown in Fig. 4, rapamycin dose-dependently restored the cell viability of MCF-7 that had been reduced by rapamycin. These findings were clear evidence that the endogenous expression of VEGF plays an essential role in rapamycin-mediated cell reduction.

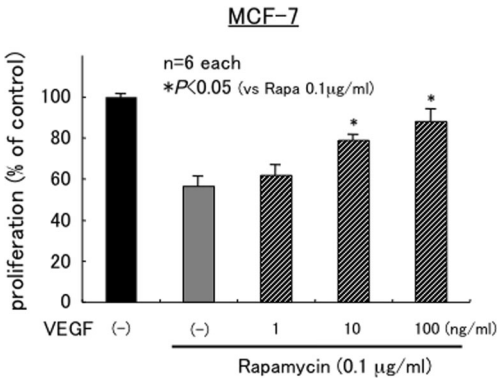

Figure 4. Rapamycin dose-dependently restored cell viability of MCF-7 reduced by rapamycin. Twenty-four hours after pre-incubation under the serum-free conditions, MCF-7 was stimulated by VEGF (0, 1, 10, 100 ng/ml) with rapamycin (100 ng/ml). Twenty-four hours later, cell viability was determined via MTT assay. *P<0.05.

Discussion

The key observations made in this study can be summarized as follows: (1) administration of rapamycin dose-dependently reduced the cell viability of MCF-7, but did not reduce the cell viability of HT-29; (2) rapamycin reduced VEGF expression in the culture medium of MCF-7, while rapamycin did not contribute VEGF expression to the culture medium of HT-29; (3) VEGF stimulated cell viability and VEGF inhibition reduced cell viability of MCF-7; and (4) rapamycin dose-dependently restored the cell viability of MCF-7 reduced by rapamycin. These results demonstrate the critical role of VEGF in the action of rapamycin as an anticancer agent on MCF-7.

Activation of the PI3K/Akt/mTOR signaling pathway has been suggested to contribute to the development of many cancers, including breast cancer (Bjornsti and Houghton 2004; Boulay *et al.* 2005). An emerging mediator of PI3K/Akt activities relating to tumor cell growth and proliferation is mTOR kinase (Bjornsti and Houghton 2004; Boulay *et al.* 2005). The importance of mTOR signaling in tumor biology is now widely accepted, and a number of agents that selectively target mTOR are being developed in cancer therapy (Bjornsti and Houghton 2004; Borders *et al.* 2010). On the other hand, it has been demonstrated that mTOR acts as an angiogenic agent (Guba *et al.* 2002; Tsutsumi *et al.* 2004; Bufalo *et al.* 2006). Recent studies have revealed that mTOR associates with VEGF expression and have suggested that mTOR inhibitors may reduce expression of VEGF in tumors (Guba *et al.* 2002; Tsutsumi *et al.* 2004; Bufalo *et al.* 2006). In the present study, rapamycin reduced VEGF expression in MCF-7, but not in HT-29. Previous reports demonstrated that the mTOR inhibitor inhibited VEGF production in vitro under both normoxic and hypoxic conditions in breast cancer (Bufalo *et al.* 2006). Several studies demonstrate that VEGF induces tumor cell proliferation and induces the survival of tumor cells through VEGF receptors or by acting directly against tumor cell apoptosis (Bachelder *et al.* 2001; Barr *et al.* 2008; Perrot-Applanat and Benedetto 2012). Therefore, VEGF is thought to be an autocrine survival factor for breast cancer cells (Bachelder *et al.* 2001; Barr *et al.* 2008; Perrot-Applanat and Benedetto 2012); this hypothesis is compatible with our current findings. One important advance of the current study is that it clarifies the necessity of VEGF in rapamycin-mediated antitumor activity in MCF-7, as the

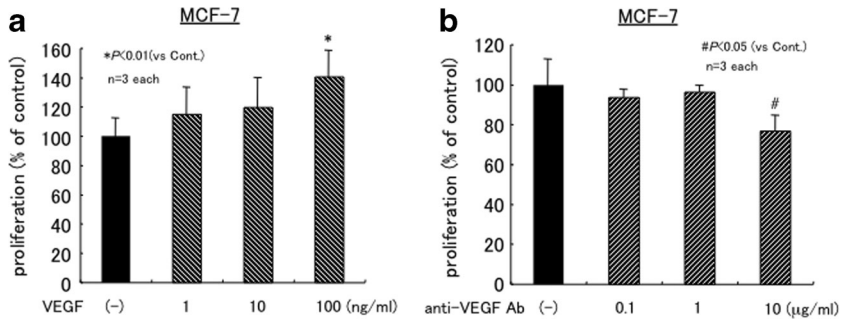

Figure 3. (*a*) VEGF dose-dependently stimulated cell viability of MCF-7. Twenty-four hours after preincubation in a serum-free condition, MCF-7 was stimulated by VEGF (0, 1, 10, 100 ng/ml). Twenty-four hours later, cell viability was determined via MTT assay. *P<0.05. (*b*) Anti-VEGF neutralizing antibody significantly reduced cell viability of MCF-7. Twenty-four hours after preincubation under the serum-free conditions, MCF-7 was treated with anti-VEGF neutralizing antibody (0, 0.1, 1, or 10 μg/ml). Twenty-four hours later, cell viability was determined via MTT assay. *P<0.05.

antitumor effect of rapamycin was reduced by neutralization of endogenous VEGF activity.

On the other hand, although mTOR inhibition is involved in antitumor activity in MCF-7, mTOR inhibition does not cause antitumor activity in HT-29. Rapamycin did not contribute to VEGF expression in HT-29; this finding also supports the hypothesis that VEGF contributes to the mTOR inhibitor-induced antitumor effect. In HT-29, the expression of VEGF is thought to be dependent on another mechanism that is independent of mTOR signaling.

The present study had certain limitations. In this study, we investigated the direct effect of rapamycin on tumor cells, but epithelial-mesenchymal interactions also play a fundamental role in cancer progression (Arias 2001). Several growth factors, including VEGF, have been revealed as key regulators of epithelial-mesenchymal interactions (Arias 2001), and it is thought that VEGF might play a significant role in the complex interplay of epithelial and mesenchymal interactions during cancer progression. One important advance of the current study was to clarify the necessity of VEGF in the rapamycin-mediated antitumor activity in MCF-7. Further studies, including in vivo studies, are needed to assess the definitive mechanism of the mTOR-inhibitor-induced antitumor effect.

In conclusion, we have demonstrated that rapamycin acts as a direct anticancer agent, and the rapamycin-induced anticancer effect was suggested to involve the reduced expression of VEGF in the breast cancer cell line MCF-7. Therefore, our results imply that mTOR regulates the VEGF expression of MCF-7 cells and is involved in breast cancer progression. An improvement in our understanding of antitumor mechanisms induced by mTOR inhibition could provide useful therapeutic targets for the treatment of not only breast cancer but also various cancers in which mTOR regulates VEGF expression.

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Competing Interests The authors declare that they have no competing interests.

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