Anti-tumor effect of recombinant plasminogen kringle 1–3 in KB cells: Histological observation and immunoprecipitation-based high performance liquid chromatography

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(Received Jan 31, 2017; Accepted Mar 3, 2017)

ABSTRACT

Angiostatin containing plasminogen kringle 1-4 domain is an endogenous angiogenesis inhibitor with anti-tumor effect. The present study applied recombinant human plasminogen kringle 1-3 (rPK1-3) in KB cell culture and BALB/c-nude mice, to elucidate the *in vitro* and *in vivo* anti-tumorigenic effect. Nude mice (n = 4) treated with rPK1-3, were compared to cisplatin treated nude mice (n = 4) and saline controls (n = 2). *In vitro*, the KB cells were also exposed to 10 μ M of rPK1-3 for 12 and 24 hours. Cells were evaluated histologically, and their protein expression was analyzed by immunoprecipitation-based high performance liquid chromatography (IP-HPLC). Results revealed that rPK1-3 potently inhibited the growth of KB cells in cell culture, but not in animal study. The KB cells treated with rPK1-3 showed growth arrest with decreased expression of PCNA, Ki-67, and MPM2, but enhanced apoptosis and increased expression of p53, BID, BAK, BAX, and caspase 9. Reduced cellular adaptation was also observed, with decreased expression of TGF- β 1, SMAD4, pAKT, mTOR, EGFR, and PKC. Anti-angiogenesis effects with decreased expression of VEGF-A, VEGFR, angiogenin, vWF, LYVE-1, D2-40, CD31, MMP-2, and bFGF were also observed in cells treated with rPK1-3. These results suggest that *in vitro*, rPK1-3 has potent anti-tumorigenic effects, such as growth inhibition, increased apoptosis, reduced cellular survival, and tumor anti-angiogenesis. Therefore, rPK1-3 can be used as an anti-tumor agent for tumor cells in the absence of host systemic responses, including antibody formation.

KEY WORDS: Angiostatin, Immunoprecipitation-based high performance liquid chromatography (IP-HPLC), KB cells, Plasminogen

Introduction

Angiogenesis is a biological process that forms new blood vessels from existing vascular channels. It is induced in situations such as body development, wound healing, and tumor growth [1-3]. When angiogenesis begins, the influ-

ence of angiogenesis stimulators is greater than that of angiogenesis inhibitors in tissues [4]. Angiogenesis stimulators such as vascular endothelial growth factor (VEGF) and angiogenin can activate vascular endothelial cells for proliferation, migration, and tube formation [5, 6], whereas angiogenesis inhibitors such as angiostatin and endostatin can inactivate vascular endothelial cells [7]. Angiogenesis is necessary for the growth of solid tumors via more expression of angiogenesis stimulators than angiogenesis inhibitors during tumor progression [8, 9]. Thus, increase of angiogenesis inhibitors or decrease of angiogenesis stimulators could be considered as a strategy to treat cancer [10].

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Angiostatin contains kringle 1-4 domains of plasminogen. It is an endogenous angiogenesis inhibitor [11]. Angiostatin is generally regarded as a direct vascular endothelial cell inhibitor. It can induce endothelial cell apoptosis and inhibit the proliferation and migration of endothelial cells [12, 13]. Theoretically, angiostatin should be able to inhibit the growth of solid tumors through anti-angiogenesis. In animal studies, angiostatin has shown anti-tumor effects mainly on tumors of endothelial origin by specific treatment modalities such as gene therapy or combination with other drugs [14-19]. However, systemic administration of angiostatin peptide alone has induced weak or no anti-tumor effect *in vivo* [20]. The clinical efficacy of angiostatin in treatment of malignant tumors has not been proven to be sufficient [21, 22]. The reason for its failure in clinical trial might be partly due to host reaction such as producing antibodies against kringle domain peptides [22].

The present study used recombinant human plasminogen kringle 1-3 (rPK1-3) produced from *Escherichia coli* [23]. Previous studies have reported that rPK1-3 can inhibit the proliferation of bovine capillary endothelial cell *in vitro* and suppress the growth of malignant tumors such as brain tumor, lung carcinoma, and melanoma *in vivo* [23, 24]. It can also inhibit the formation of new blood vessels in rabbit cornea models [25, 26]. In this study, rPK1-3 was tested on KB cell (HELA contaminant human epithelial carcinoma). Epithelial origin cancer is the most common type of cancer. However, few information is available about the effect of rPK1-3 on KB cell. Therefore, the objective of this study was to determine the anti-tumor effect of rPK1-3 in KB cell (*in vitro*) and BALB/c-nude mouse (*in vivo*). In addition, protein expression changes of KB cell after treatment with rPK1-3 were analyzed by immunoprecipitation-based high performance liquid chromatography (IP-HPLC), a method recently developed to accurately detect protein expression levels with minimum error range (less than 5%). IP-HPLC analysis is partly similar to enzyme-linked immunosorbent assay (ELISA). However, it can produce more precise quantitative data than ELISA [27].

Materials and methods

Recombinant human plasminogen kringle 1–3 domains

The recombinant human plasminogen kringle 1-3 (rPK1-3) contained 254 amino acids. It was produced from *Esche-*

richia coli by purification process and kindly provided by Dr. Soo-Il Chung from Mogam Biotechnology Research Institute [23].

Cell culture

Frozen KB cell (HELA contaminant human epithelial carcinoma) was obtained from Korean Cell Line Bank (Seoul, Republic of Korea). After thawing, KB cells were maintained as monolayer culture in RPMI-1640 medium (HyClone, UT, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, CA, USA) and penicillin-streptomycin (Lonza Walkersville, MD, USA), in 5% CO₂ at 37°C.

Animal experiment

Six-week-old male BALB/c-nude mice were purchased from Orient Bio Co. (Seoul, Korea). They were used in accordance with animal care and use guidelines of the College of Dentistry at Gangneung-Wonju National University (GWNU-2013-34). To produce tumors, cultured KB cells were suspended in culture medium and injected into the back of nude mice subcutaneously. Nude mice were randomly assigned into three groups: rPK1-3 treatment group (n = 4), cisplatin treatment group (n = 4) as positive control, and saline treatment group (n = 2) as negative control. After tumor mass was conspicuously found in gross observation, each drug treatment was started by peritoneal injection twice a week. The dosages were: 2 mg/kg of cisplatin (Choongwae, Seoul, Korea), 100 mg/kg of rPK1-3, and 0.2 ml of normal saline [24]. Changes in tumor mass size and body weight were measured twice a week for seven weeks. Tumor mass size was calculated using the following formula: mass volume = a (long distance) × b (short distance)² / 2 [28].

Histological observation and tumor cell counting

KB cells were cultured on cell culture petri dishes for experimental group and control groups separately. The experimental group was treated with rPK1-3 at a final concentration of 10 μM whereas the control group was untreated. After 12 and 24 hours of culture for each group, KB cells were fixed with 10% neutral formaldehyde solution and stained with hematoxylin and eosin. KB cells from each group were observed under microscope. Images were captured with a digital camera (DP-70®, Olympus Co., Japan). Representative images (n = 8) in high magnification (×400) were subjected to cell counting using

IMT i-solution version 21.1 (Vancouver, Canada). Statistical analysis was performed with IBM SPSS statistics 19.0 (SPSS Inc., Chicago, IL, USA). Statistical significance level was set at $P < 0.05$.

Immunoprecipitation-based high performance liquid chromatography (IP-HPLC)

After subculture, KB cells were divided into experimental group and control group. During the active growth period, the experimental group was treated with rPK1-3 at a final concentration of 10 μM for 24 hours while the control group was not treated. About 1×10^{10} KB cells were harvested per each group and immediately lysed with protein extraction buffer (PRO-PREPTM, iNtRON Biotech., USA) on ice. Protein extracts from experimental and control groups were centrifuged at 1,600 g for 30 min. Supernatants were collected and stored in a deep freezer at -70°C until use. Protein concentrations of supernatants were measured with a protein assay solution (Bio-Rad Laboratories, Inc., USA) at wavelength of 592 nm using a UV spectrophotometer.

Each 100 μg of protein extract was subjected to immu-

noprecipitation using protein A/G agarose column (Amicogen, Korea). Protein A/G agarose columns were separately pre-incubated with 1 μg of 41 different antisera, including β -actin, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), PCNA (proliferating cell nuclear antigen), Ki-67 (antigen KI-67), MPM2 (mitotic protein monoclonal 2), CDK4 (cyclin-dependent kinase 4), p16, p21, RB-1 (retinoblastoma protein), p53, BID (BH3 interacting-domain death agonist), BAK (Bcl-2 homologous antagonist/killer), BAX (Bcl-2-associated X protein), BAD (Bcl-2-associated death promoter), BCL2 (B-cell lymphoma 2), caspase 9, TGF- β 1 (transforming growth factor beta 1), SMAD4 (mothers against decapentaplegic homolog 4), pAKT (phosphorylated protein kinase B), mTOR (mechanistic target of rapamycin), NF κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), IKK (I κ B kinase), EGFR (epidermal growth factor receptor), HER2 (human epidermal growth factor receptor 2), IGFIIR (insulin-like growth factor 2 receptor), HGF (hepatocyte growth factor), survivin, hTERT (telomerase reverse transcriptase), HO-1 (heme oxygenase 1), FAK (focal adhesion kinase), PKC (protein kinase C), leptin, VEGF-A (Vascular endo-

Fig. 1. Representative chromatography overlap between rPK1-3 treatment group and the control group. Peak areas of pAKT and VEGFR were bigger in rPK1-3 treatment group than those in the control group. However, peak area of leptin was smaller in the rPK1-3 treatment group than that in the control group.

thelial growth factor A), VEGFR (vascular endothelial growth factor receptor), angiogenin, vWF (von Willebrand factor), LYVE-1 (Lymphatic vessel endothelial hyaluronan receptor 1), D2-40 (podoplanin), CD31 (cluster of differentiation 31), MMP-2 (matrix metalloproteinase-2), and bFGF (Basic fibroblast growth factor).

Briefly, protein samples were mixed with 5 mL of binding buffer (150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF and 0.5% NP-40) and incubated in protein A/G agarose columns at 10°C for 1 hour. The columns were placed on a rotating stirrer during incubation. After washing each column with sufficient amount of PBS solution (pH 7.3, 137 mM NaCl, 2.7 mM KCl, 43 mM Na₂HPO₄·7H₂O and 1.4 mM KH₂PO₄), target protein was eluted with 250 µL IgG elution buffer (Pierce, USA). Immuno-precipitated proteins were analyzed by HPLC (1100 series, Agilent, USA) using a reverse phase column (YMC-Pack SIL, Japan) running with 0.15M NaCl and 20% acetonitrile solution at 0.4 mL/min for 30 min. They were analyzed by UV spectroscopy at 280 nm. Both control and experimental groups were simultaneously performed for IP-HPLC. In the results of IP-HPLC, sample protein peak areas (mAU*s) obtained from HPLC analysis were eliminated by antibody peak area (mAU*s) in the negative control. Data were mathematically reduced into square root value as the unit level for molecular concentration and subsequently compared to each other between the experimental group and the control group (Fig. 1). All square root values of protein peak areas were plotted into a graph depending on the characteristics of proteins.

Results

Animal experiment

A total of ten nude mice were implanted with KB cells. Subsequently they were treated with rPK1-3 for the experimental group, cisplatin for the positive control group, and normal saline for the negative control group. Tumor sizes of all groups were gradually increased until 49 days after the initial treatment. At the first two weeks after the initial treatment with rPK1-3, similar tumor size was found compared to other groups. However, tumor size of the rPK1-3 treated group was increased rapidly thereafter. It became larger than other groups from five to seven weeks.

Regarding body weight changes, there was no signifi-

Fig. 2. Effect of rPK1-3 on the KB cell xenografted nude mice. A: Tumor size change during 49 days after the initial treatment. B: Body weight change during 49 days after the initial treatment.

cant difference among the three groups. However, the saline treated group showed the highest weight increase at seven weeks compared to the other two groups while rPK1-3 treated group and cisplatin treated group showed similar weight increases at seven weeks after the initial treatment (Fig. 2).

KB Cell counting in KB cell culture

The number of KB cells after treatment with rPK1-3 was decreased to 107.8 ± 31.6 after 12 hours and 94.6 ± 7.6 after 24 hours compared to 139.5 ± 22.5 after 12 hours and 164.6 ± 35.9 after 24 hours in the control group in (Fig. 3). The number of control KB cells was consistently increased during 24 hours. However, the number of KB cells after rPK1-3 treatment was decreased during 24 hours. The difference in cell number between rPK1-3 treated and control was significant ($P < 0.05$) at both 12 and 24 hours (Fig. 3).

Histological observation of KB cell culture

In histological observation, KB cells treated with rPK1-3 showed different features of cellular differentiation compared to untreated control cells. Control KB cells were dispersed on the petri dish surface. They grew actively

Fig. 3. A graph showing the results of cell number counting for rPK1-3 treated KB cells and untreated control cells. Treatment with rPK1-3 decreased the number of KB cells to 107.8 ± 31.6 after 12 hours and 94.6 ± 7.6 after 24 hours compared to 139.5 ± 22.5 after 12 hours and 164.6 ± 35.9 after 24 hours in the control group. Control KB cells were consistently increased in number, while rPK1-3 treated KB cells were decreased in number during 24 hours. The difference in cell number between rPK1-3 treated KB cells and controls was significant at both 12 hours ($P = 0.038$) and 24 hours ($P < 0.01$).

with long cytoplasmic process at 12 hours after culturing (Fig. 4A1). They were highly proliferative in spindle to dendritic shapes with hyperchromatic and pleomorphic nuclei (Fig. 4A2). However, KB cells treated with rPK1-3 were gradually aggregated. They formed epithelial clusters at 12 hours after culturing (Fig. 4B1). Some epithelial tumor cells showed abundant cytoplasm filled with keratin materials similar to normal squamous keratinocytes (Fig. 4B2). After 24 hours of culturing, control KB cells showed overgrowth on the petri dish surface (Fig. 4C1). Their nuclei were consistently hyperchromatic and pleomorphic (Fig. 4C2). However, KB cells treated with rPK1-3 grew slowly on the petri dish surface. They formed aggregated epithelial clusters (Fig. 4D1). Many tumor cells became enlarged with abundant cytoplasm. They gradually attached to each other, resembling differentiation of normal squamous epithelium (Fig. 4D2).

Immunoprecipitation-based high performance liquid chromatography (IP-HPLC)

Protein expressions in rPK1-3 treated KB cell were assessed through IP-HPLC analysis compared to those of untreated control. In terms of proliferation and apoptosis, rPK1-3 treated KB cells showed decreased expression of β -actin (98.9%), GAPDH (96.1%), PCNA (79%), Ki-67 (91.1%), MPM2 (90.0%), P16 (96.5%), and BAD (72.8%) but increased expression of CDK4 (115.5%), p21 (140.0%), RB-1 (104.1%), p53 (124.5%), BID (107.6%), BAK (109.1%),

Fig. 4. Photomicrographs of rPK1-3 treated KB cells. A and C: Untreated control. B and D: KB cells treated with rPK1-3 at 12 h and 24 h. Control KB cells were dispersed on petri dish surface. They grew actively with long cytoplasmic processes at 12 hours after culture (A1). They were highly proliferative in spindle to dendritic shape with hyperchromatic and pleomorphic nuclei (A2). KB cells treated with rPK1-3 were gradually aggregated. They formed epithelial clusters at 12 hours after culture (B1). Some epithelial tumor cells showed abundant cytoplasm filled with keratin materials, similar to normal squamous keratinocytes (B2, arrows). At 24 hours after culture, control KB cells showed overgrowth on the petri dish surface (C1). Their nuclei were consistently hyperchromatic and pleomorphic (C2). KB cells treated with rPK1-3 grew slowly on the petri dish surface, forming aggregated epithelial clusters (D1). Many tumor cells became enlarged with abundant cytoplasm. They gradually attached to each other, resembling differentiation of normal squamous epithelium (D2, arrows).

BAX (111.8%), BCL2 (104.1%), and caspase 9 (122.6%) compared to untreated control cells (Fig. 5).

In terms of cellular adaptation for survival, rPK1-3-treated KB cells showed decreased expression of TGF- β 1 (80.3%), SMAD4 (97.4%), pAKT (77.8%), mTOR (90.5%), NF κ B (99.2%), EGFR (92.6%), survivin (83.3%), HO-1 (99.2%), FAK (99.7%), and PKC (91.3%) but increased

Fig. 5. A graph plotted with data of IP-HPLC analysis. KB cells treated with rPK1-3 showed growth inhibition with decreased expression of PCNA, Ki-67, and MPM2 but increased expression of p21. Expression levels of CDK4 and RB-1 were increased compensatory to growth inhibition of KB cells. They also underwent cellular apoptosis with increased expression of p53, BID, BAK, BAX, and caspase 9. BCL2 was slightly increased compensatory to enhanced apoptosis. However, the expression of FAS-related apoptosis protein BAD was significantly decreased. On the other hand, expression levels of ordinary cellular keeping proteins such as β -actin and GAPDH were decreased only slightly.

Fig. 6. A graph plotted with data of IP-HPLC analysis. KB cells treated with rPK1-3 showed decreased potential of cellular adaptation for survival with decreased expression of TGF- β 1, SMAD4, pAKT, mTOR, EGFR, and PKC, resulting in inhibition of cellular survival with decreased expression of survivin. Although there were no significant changes in the expression of cellular stress proteins including NFkB, IKK, HO-1, and FAK, the expression levels of IGFIIR, HGF, hTERT, and leptin were increased which might be compensatory to reduced cellular adaptation.

Fig. 7. A graph plotted with data of IP-HPLC analysis. KB cells treated with rPK1-3 showed antiangiogenesis effect with decreased expression of VEGF-A, VEGFR, angiogenin, vWF, LYVE-1, D2-40, CD31, MMP-2, and bFGF.

expression of IKK (104.5%), HER2 (107.3%), IGFIIR (103.5%), HGF (106.4%), hTERT (112.6%), and leptin (108.0%) compared to untreated control cells (Fig. 6).

In terms of angiogenesis, rPK1-3-treated KB cells showed decreased expression of VEGF-A (93.7%), VEGFR (87.3%), angiogenin (91.9%), vWF (81.5%), LYVE-1 (91.3%), D2-40 (90.4%), CD31 (89.7%), MMP-2 (92.6%), and bFGF (86.2%) compared to untreated control cells (Fig. 7).

Discussion

Plasminogen includes kringle 1-5 domains and kringle domain. Domains of plasminogen mostly inhibit the proliferation and migration of endothelial cells. Kringle domain peptides are known to have anti-angiogenic properties [11]. The rPK1-3 includes kringle 1-3 domains of plasminogen. Anti-angiogenesis through anti-endothelial activities is the main mechanism of kringle domain peptides of plasminogen in malignant tumors. However, the detailed mechanism involved in the anti-angiogenesis effect of kringle domain peptides still remains unclear. In this study, the antiangiogenic and anti-tumor effects of rPK1-3 were observed in KB cell-xenograft animal and KB cell culture. In addition, protein expression changes caused by rPK1-3 treatment were determined.

The present animal study showed that there was no significant difference among the three groups regarding changes in tumor size or body weight (Fig. 2). Tumor sizes of all groups were gradually increased during 49 days with minor difference among the three groups. Cisplatin-treated group showed the lowest growth rate of tumor mass while rPK1-3 treated group showed the highest growth rate of tumor mass among the three groups. The present study clearly demonstrated that rPK1-3 treatment for 7 weeks *in vivo* had non-effect on tumor mass.

In terms of dosage and duration of rPK1-3, other xenograft studies have reported that rPK1-3 has favorable therapeutic outcomes as anti-cancer therapy by administration of 100 mg/kg of rPK1-3 a day for 3 weeks [23, 24]. Our study applied the same dose. However, we only administered rPK1-3 twice a week for 7 weeks. A relatively long term treatment of rPK1-3 for 7 weeks might have induced host immune response compared to treatment for 3 weeks. The observed no anti-tumor effect of rPK1-3 in our *in vivo* experiment might be caused by antibody production against rPK1-3 peptide in nude mouse via T-cell independent B-

cell activation. BALB/c nude mouse is an athymic mouse without T cell function. However, its B cell immunity is still active. The rPK1-3 peptide might be big enough to be strongly antigenic to host immune system for the production of antibody [29]. This hypothesis could be supported by the fact that some peptides containing repetitive amino acid sequences such as bacterial proteins and plasminogen kringle domains can directly induce the production of antibody due to B-cell stimulation without help of T-cells in nude mouse [30, 31]. Similarly, it has been reported that endostatin, an inhibitor protein of angiogenesis, can induce the production of antibody in some cancer patient, thus causing clinical failure of anti-angiogenesis therapy of endostatin [32].

In cell culture study, KB cells treated with rPK1-3 showed inhibition in cellular proliferation compared to untreated control (Fig. 3). They also showed more cellular differentiation compared to untreated control. Cell aggregation and cytoplasm enlargement were more prominent in rPK1-3-treated KB cells than those in untreated controls (Fig. 4). IP-HPLC analysis results supported the suppression of KB cell proliferation by rPK1-3. KB cells treated with rPK1-3 also showed growth inhibition with decrease expression of PCNA, Ki-67, and MPM2 but increased expression of p21 with compensatory increase of CDK4 and RB-1. They also underwent cellular apoptosis with increased expression of p53, BID, BAK, BAX, and caspase 9. BCL2 was slightly increased with compensatory enhancement of apoptosis. However, FAS-related apoptosis protein BAD was severely decreased in expression. On the other hand, the expression levels of ordinary cellular keeping proteins such as β -actin and GAPDH were slightly decreased (Fig. 5). Because IP-HPLC analysis can provide precise results with scientifically significant error range (less than 5%), the present study clearly demonstrated increased apoptosis signaling compared to anti-apoptosis signaling after treatment with rPK1-3.

KB cells treated with rPK1-3 also showed reduced cellular adaptation with decreased expression of TGF- β 1, SMAD4, pAKT, mTOR, EGFR, and PKC, resulting in inhibition of cellular survival with decreased expression of survivin. Although there were no significant expression changes of cellular stress proteins including NF κ B, IKK, HO-1, and FAK, the expression levels of IGFIIR, HGF, hTERT, and leptin were increased which might be compensatory to reduced cellular adaptation (Fig. 6).

The above results might directly indicate that rPK1-3

could suppress the proliferation and survival of KB cells. Particularly, the expression levels of known representative cell proliferation markers such as PCNA, Ki-67, EGFR, and survivin [33-36] were down-regulated by rPK1-3 treatment. The over-expression of these proteins might be related to tumor aggressiveness and poor prognosis of several malignant tumors [36-38]. In addition, tumor suppressor proteins such as p53, p21, and RB-1 [39-41] were down-regulated by rPK1-3, thus inducing tumor cell apoptosis and cell cycle suppression. It is known that p53 is involved in DNA repair and cell apoptosis to maintain tissue stability after cell damage [39]. It has been reported that p21 expression is induced by p53 and that p21 interacts with PCNA [42]. The remarkable up-regulation of p53 and p21 but down-regulation of PCNA by rPK1-3 suggested that rPK1-3 had anti-proliferative effect in KB cells in this study (Fig. 5). As tumor cells grow, they require enough angiogenesis to supply nutrients and oxygen for their survival. Angiogenesis occurs more actively in advanced malignant tumors [9]. In the present IP-HPLC study, angiogenesis-related proteins such as VEGF-A, VEGFR, angiogenin, vWF, LYVE-1, D2-40, CD31, MMP-2, and bFGF were consistently down-regulated by the rPK1-3 treatment (Fig. 7). It is known that VEGF-A, VEGFR, and angiogenin are potent inducers of angiogenesis [43, 44]. CD31 has function for endothelial cell adhesion by localizing at the intercellular junction of vascular endothelial cells [45]. bFGF is an important wound healing factor to support the angiogenesis process [3]. Similar results have been reported showing that rPK1-3 treatment can down-regulate the expression of VEGF and bFGF in malignant tumor xenograft in an *in vivo* study [24]. However, the fact that all angiogenesis-related proteins were dramatically down-regulated by rPK1-3 peptide treatment in the present KB cell culture experiment, identical to previous other studies, supported that rPK1-3 peptide might have potent anti-angiogenic and anti-tumor effect when it is directly applied to tumor cells.

Conclusions

In *in vitro* KB cell culture experiment, rPK1-3 clearly showed strong anti-tumor effect via anti-angiogenesis and anti-proliferation effects. The rPK1-3 peptide showed important roles in growth inhibition, increased apoptosis, reduced cell survival of KB cells in *in vitro*. However,

these effects were not observed in *in vivo* condition. Therefore, rPK1-3 might be applied directly to tumor cells to exhibit its potent anti-tumor effect in the absence of host systemic responses including antibody formation.

Acknowledgements

We are deeply grateful to Dr. Soo IL Chung who kindly provided rPK1-3 peptide and great suggestions. This work was supported by the Cooperative Research (CR1301) of Gangneung-Wonju National University Dental Hospital.

Conflict of Interest

The authors declare that they have no competing interests.

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References

1. Sauer H, Günther J, Hescheler J, Wartenberg M. Thalidomide inhibits angiogenesis in embryoid bodies by the generation of hydroxyl radicals. *Am J Pathol* 2000;156:151-158. doi: 10.1016/S0002-9440(10)64714-1.
2. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407:249-257. doi: 10.1038/35025220.
3. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. *J Invest Dermatol Symp Proc* 2000;5:40-46. doi: 10.1046/j.1087-0024.2000.00014.x.
4. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003;3:401-410. doi: 10.1038/nrc1093.
5. Gao X, Xu Z. Mechanisms of action of angiogenin. *Acta Biochim Biophys Sin* 2008;40:619-624. doi: 10.1111/j.1745-7270.2008.00442.x.
6. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;9:669-676. doi: 10.1038/nm0603-669.
7. Cao Y. Endogenous angiogenesis inhibitors: angiostatin, endostatin, and other proteolytic fragments. *Prog Mol*

- Subcell Biol 1998;20:161-176.
8. Folkman J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 2002;29:15-18. doi: 10.1016/S0093-7754(02)70065-1.
9. Eichhorn ME, Kleespies A, Angele MK, Jauch KW, Bruns CJ. Angiogenesis in cancer: molecular mechanisms, clinical impact. *Langenbecks Arch Surg* 2007;392:371-379. doi: 10.1007/s00423-007-0150-0.
10. Cao Y. Antiangiogenic cancer therapy. *Semin Cancer Biol* 2004;14:139-145. doi: 10.1016/j.semcancer.2003.09.018.
11. Cao Y, Xue L. Angiostatin. *Semin Thromb Hemost* 2004;30:83-93. doi: 10.1055/s-2004-822973.
12. Lucas R, Holmgren L, Garcia I, Jimenez B, Mandriota SJ, Borlat F, Sim BK, Wu Z, Grau GE, Shing Y, Soff GA, Bouck N, Pepper MS. Multiple forms of angiostatin induce apoptosis in endothelial cells. *Blood* 1998;92:4730-4741.
13. Claesson-Welsh L, Welsh M, Ito N, Anand-Apte B, Soker S, Zetter B, O'Reilly M, Folkman J. Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. *Proc Natl Acad Sci USA* 1998;95:5579-5583.
14. Kirsch M, Strasser J, Allende R, Bello L, Zhang J, Black PM. Angiostatin suppresses malignant glioma growth in vivo. *Cancer Res* 1998;58:4654-4659.
15. Lannutti BJ, Gately ST, Quevedo ME, Soff GA, Paller AS. Human angiostatin inhibits murine hemangioendothelioma tumor growth in vivo. *Cancer Res* 1997;57:5277-5280.
16. Yokoyama Y, Dhanabal M, Griffioen AW, Sukhatme VP, Ramakrishnan S. Synergy between angiostatin and endostatin: inhibition of ovarian cancer growth. *Cancer Res* 2000;60:2190-2196.
17. Scappaticci FA, Smith R, Pathak A, Schloss D, Lum B, Cao Y, Johnson F, Engleman EG, Nolan GP. Combination angiostatin and endostatin gene transfer induces synergistic antiangiogenic activity in vitro and antitumor efficacy in leukemia and solid tumors in mice. *Mol Ther* 2001;3:186-196. doi: 10.1006/mthe.2000.0243.
18. Ponnazhagan S, Mahendra G, Kumar S, Shaw DR, Stockard CR, Grizzle WE, Meleth S. Adeno-associated virus 2-mediated antiangiogenic cancer gene therapy: long-term efficacy of a vector encoding angiostatin and endostatin over vectors encoding a single factor. *Cancer Res* 2004;64:1781-1787. doi: 10.1158/0008-5472.
19. Indraccolo S, Morini M, Gola E, Carrozzino F, Habeler W, Minghelli S, Santi L, Chieco-Bianchi L, Cao Y, Albini A, Noonan DM. Effects of angiostatin gene transfer on functional properties and in vivo growth of Kaposi's sarcoma cells. *Cancer Res* 2001;61:5441-5446.
20. Bergers G, Javaherian K, Lo KM, Folkman J, Hanahan D. Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. *Science* 1999;284:808-812. doi: 10.1126/science.284.5415.808.
21. Kurup A, Lin CW, Murry DJ, Dobrolecki L, Estes D, Yiannoutsos CT, Mariano L, Sidor C, Hickey R, Hanna N. Recombinant human angiostatin (rhAngiostatin) in combination with paclitaxel and carboplatin in patients with advanced non-small-cell lung cancer: a phase II study from Indiana University. *Ann Oncol* 2006;17:97-103. doi: 10.1093/annonc/mdj055.
22. Beerepoot LV, Witteveen EO, Groenewegen G, Fogler WE, Sim BK, Sidor C, Zonnenberg BA, Schramel F, Gebbink MF, Voest EE. Recombinant human angiostatin by twice-daily subcutaneous injection in advanced cancer: a pharmacokinetic and long-term safety study. *Clin Cancer Res* 2003;9:4025-4033.
23. You WK, So SH, Sohn YD, Lee H, Park DH, Chung SI, Chung KH. Characterization and biological activities of recombinant human plasminogen kringle 1-3 produced in *Escherichia coli*. *Protein Expr Purif* 2004;36:1-10. doi: 10.1016/j.pep.2004.02.019.
24. Joe YA, Hong YK, Chung DS, Yang YJ, Kang JK, Lee YS, Chang SI, You WK, Lee H, Chung SI. Inhibition of human malignant glioma growth in vivo by human recombinant plasminogen kringles 1-3. *Int J Cancer* 1999;82:694-699.
25. Shin SH, Kim JC, Chang SI, Lee H, Chung SI. Recombinant kringle 1-3 of plasminogen inhibits rabbit corneal angiogenesis induced by angiogenin. *Cornea* 2000;19:212-217.
26. Kim JH, Kim JC, Shin SH, Chang SI, Lee HS, Chung SI. The inhibitory effects of recombinant plasminogen kringle 1-3 on the neovascularization of rabbit cornea induced by angiogenin, bFGF, and VEGF. *Exp Mol Med* 1999;31:203-209. doi: 10.1038/emmm.1999.33.
27. Kim SM, Eo MY, Cho YJ, Kim YS, Lee SK. Differential protein expression in the secretory fluids of maxillary sinusitis and maxillary retention cyst. *Eur Arch Otorhinolaryngol* 2017;274:215-222. doi: 10.1007/s00405-016-4167-2.
28. Carlsson G, Gullberg B, Hafström L. Estimation of liver tumor volume using different formulas - an experimental study in rats. *J Cancer Res Clin Oncol* 1983;105:20-23. doi:10.1007/BF00391826.
29. Dell'Eva R, Pfeffer U, Indraccolo S, Albini A, Noonan D. Inhibition of tumor angiogenesis by angiostatin: from recombinant protein to gene therapy. *Endothelium* 2002;9:3-10. doi: 10.1080/10623320210712.
30. Rüde E, Wrede J, Gundelach ML. Production of IgG antibodies and enhanced response of nude mice to DNP-AE-dextran. *J Immunol* 1976;116:527-533.
31. Hillman K, Shapira-Nahor O, Blackburn R, Hernandez D, Golding H. A polymer containing a repeating peptide sequence can stimulate T-cell-independent IgG antibody

- production in vivo. *Cell Immunol* 1991;134:1-13. doi: 10.1016/0008-8749(91)90326-7.
32. Bachelot T, Ratel D, Menetrier-Caux C, Wion D, Blay JY, Berger F. Autoantibodies to endostatin in patients with breast cancer: correlation to endostatin levels and clinical outcome. *Br J Cancer* 2006;94:1066-1070. doi: 10.1038/sj.bjc.6603037.
33. Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. *Cell* 2007;129:665-679. doi: 10.1016/j.cell.2007.05.003.
34. Bullwinkel J, Baron-Lühr B, Lüdemann A, Wohlenberg C, Gerdes J, Scholzen T. Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *J Cell Physiol* 2006;206:624-635. doi: 10.1002/jcp.20494.
35. Oda K, Matsuoka Y, Funahashi A, Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling. *Mol Syst Biol* 2005;1:2005.0010. doi: 10.1038/msb4100014.
36. Mita AC, Mita MM, Nawrocki ST, Giles FJ. Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. *Clin Cancer Res* 2008;14:5000-5005. doi: 10.1158/1078-0432.CCR-08-0746.
37. Myoung H, Kim MJ, Lee JH, Ok YJ, Paeng JY, Yun PY. Correlation of proliferative markers (Ki-67 and PCNA) with survival and lymph node metastasis in oral squamous cell carcinoma: a clinical and histopathological analysis of 113 patients. *Int J Oral Maxillofac Surg* 2006;35:1005-1010. doi: 10.1016/j.ijom.2006.07.016.
38. Wong DT, Todd R. Epidermal growth factor receptor (EGFR) biology and human oral cancer. *Histol Histopathol* 1999;14:491-500.
39. Surget S, Khoury MP, Bourdon JC. Uncovering the role of p53 splice variants in human malignancy: a clinical perspective. *Onco Targets Ther* 2014;7:57-68. doi: 10.2147/OTT.S53876.
40. Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res* 2005;65:3980-3985. doi: 10.1158/0008-5472.CAN-04-3995.
41. Murphree L, Benedict WF. Retinoblastoma: clues to human oncogenesis. *Science* 1984;223:1028-1034.
42. Waga S, Hannon GJ, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 1994;369:574-578. doi: 10.1038/369574a0.
43. Tello-Montoliu A, Patel JV, Lip GY. Angiogenin: a review of the pathophysiology and potential clinical applications. *J Thromb Haemost* 2006;4:1864-1874. doi: 10.1111/j.1538-7836.2006.01995.x.
44. Shibuya M. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. *J Biochem* 2013;153:13-19. doi: 10.1093/jb/mvs136.
45. Albelda SM, Muller WA, Buck CA, Newman PJ. Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. *J Cell Biol* 1991;114:1059-1068. doi: 10.1083/jcb.114.5.1059.