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Title	Pigment epithelium-derived factor inhibits the growth of human esophageal squamous cell carcinoma by suppressing neovascularization. [an abstract of dissertation and a summary of dissertation review]
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Description	配架番号：2128
Degree Grantor	北海道大学
Degree Name	博士(医学)
Dissertation Number	甲第11646号
Issue Date	2015-03-25
Doc URL	https://hdl.handle.net/2115/59122
Rights(URL)	https://creativecommons.org/licenses/by-nc-sa/2.1/jp/
Type	doctoral thesis
File Information	Masatoshi_Kadoya_abstract.pdf, 論文内容の要旨

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学位論文内容の要旨

博士の専攻分野の名称　博士（医　学）　氏 名　角谷　昌俊

学 位 論 文 題 名

Pigment epithelium-derived factor inhibits the growth of human esophageal squamous cell carcinoma by suppressing neovascularization.
（PEDF は血管新生を阻害することにより食道癌の増殖を抑制しうる）

【Introduction】PEDF, 50-kDa secreted glycoprotein and member of the serpin superfamily of serine protease inhibitors, was first purified from human retinal pigment epithelial cell-conditioned media. PEDF has been identified as one of the most potent inhibitors of angiogenesis. Previously, the low expression of PEDF was indicated to be associated with increased risk of hepatic metastasis and short survival by immunohistochemical analysis and that *in vivo* transfer of PEDF gene inhibited tumor growth in mice implanted with the human pancreatic adenocarcinoma cell lines. But in other carcinomas that prognosis has been still poor, the efficacies of PEDF have remained unknown. Neovascularization consists of two mechanisms of angiogenesis and vasculogenesis which bone marrow-derived endothelial progenitor cells (EPCs) contribute to new blood vessel formation. Although PEDF has been well known to be an inhibitor of angiogenesis, the effect of PEDF on vasculogenesis have not been elucidated fully. Therefore, in this experiment, the antitumor property of PEDF transferred to human ESCC, one of the poorest prognostic carcinomas, and the effect of PEDF on EPCs in the peripheral blood and tumor tissues were investigated.

【Materials and Methods】The endogenous PEDF expression level were beforehand screened by western blotting analysis and two human ESCC cell lines, one is TE8 which does not endogenously secretes PEDF and another is HEC46 which endogenously secretes PEDF, were selected. In both cell lines, in addition to original wild type cells (TE8WT, HEC46WT), PEDF-overexpressing cell line (TE8P, HEC46P) and PEDF-lacking GFP-expressing control cell line (TE8G, HEC46G) were established through lentiviral vector transduction. *In vitro* experiment, the effect of PEDF on the vascular endothelial cells proliferation and migration was examined. *In vivo* experiment, the influence of PEDF on neovascularization and tumor growth was examined in subcutaneously ESCC cells implanted mice. The subcutaneous tumors were monitored their size and the mice were sacrificed at 28 days after subcutaneous injection. To estimate the antiangiogenesis activation of PEDF, quantification of intratumoral microvessel density (MVD) was performed by immunohistochemical analysis. The antitumor efficacy of PEDF was investigated by the quantitative analyses of apoptosis using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. The ratio of TUNEL-positive area to total area (Apoptotic Area Ratio; AAR) was measured by using NIH image. Then viable tumor volume (VTV) was approximately calculated as the tumor volume at 28 days×(100-AAR)×1/100. Fluorescence activated cell sorting (FACS) analysis was performed to compare the frequency of EPCs in peripheral blood in each ESCC cells implanted model. Firstly, peripheral blood mononuclear cells (PBMCs) were extracted from the whole blood of the mouse. Successively, EPCs were evaluated by a FACS Calibur using appropriate analysis gate and EPCs number per ml peripheral blood was calculated. By fluorescence microscopic examinations, the frequency of EPCs in tumor vessels of each group was quantified by the field rate containing EPCs to total 25 fields per tissue sample extracted from six mice of each group. Statistical significance was evaluated using the Mann-Whitney U test and $P<0.05$ was considered significant.

【Results】*In vitro* study, TE8WT cells promoted the vascular endothelial cells proliferation and migration, but these were significantly inhibited by PEDF gene transfer. Meanwhile HEC46WT cells did not promote the vascular endothelial cells proliferation and migration, and these could not be suppressed by PEDF gene transfer. *In vivo* study, subcutaneous tumors implanted TE8WT and TE8G cells grew rapidly, but tumor implanted TE8P cells grew significantly slowly. MVD in tumor tissues derived from TE8P cells implanted mouse have showed a marked reduction compared with TE8WT or TE8G cells implanted mice. However there was no difference in subcutaneous tumor growth and MVD among HEC46WT, G, and P cells in spite of demonstrating PEDF overexpression in HEC46P cells by immunohistochemistry. In evaluation of apoptotic change, AAR of tumor sections from TE8P cells significantly increased than that of TE8WT and TE8G cells and VTV in tumor tissue from TE8P cells also significantly decreased than TE8WT and TE8G cells. But there was no difference in AAR and VTV among the tumors implanted HEC46WT, G, and P cells. In EPCs measurement by flow cytometry, the number of EPCs from PEDF-overexpressing cells bearing mouse were significantly smaller than WT and GFP-expressing control cells bearing mouse in both TE8 and HEC46 cells. The contributions of EPCs to tumor vessel formation were estimated by using immunofluorescence staining examination. EPCs recruitment was significantly decreased in tumor tissues implanted with PEDF-overexpressing cells than those implanted WT and GFP-expressing control cells in both TE8 and HEC46 cells.

【Discussion】Conquering ESCC is still a major clinical problem to be solved. Existing multimodality treatments including surgical resection, adjuvant chemotherapy and radiotherapy have given us unsatisfactory consequences. So development of novel therapeutic strategies for ESCC is required. Although the increasing expression level of PEDF in tumor tissues was reported to result in suppression of tumor growth and metastasis in various epithelial cancers at animal models, this study demonstrated for the first time that PEDF have the antitumor effect to human ESCC cells. In this experiment, PEDF was indicated to exhibit the antitumor effect in ESCC by inhibition of intratumoral vessels, which was caused by suppression of endothelial cell proliferation and migration, and to lead cancer cells to apoptosis. Recently, some reports indicated that PEDF not only affected to inhibit angiogenesis, but also had the ability to induce apoptosis in malignant tumors. Although the mechanism of PEDF-induced apoptosis is not still elucidated fully, PEDF certainly led carcinoma cells to apoptosis and could decrease the viable carcinoma cells in ESCC cell line not secreting endogenous PEDF. PEDF may show antitumor effect by not only inhibiting neovascularization but also inducing tumor cell apoptosis in human ESCC. But in tumor cells secreting endogenous PEDF, the apoptotic effect by PEDF gene transfer may be weak. From the result of measurement of EPCs, PEDF seemed to suppress the release of EPCs from bone marrow to peripheral bloods and successively reduce the incorporation of EPCs into tumor tissues, and result in inhibiting vasculogenesis. This fact was also showed in this study for the first time. PEDF certainly could exhibit the potent antitumor properties by inhibiting both angiogenesis and vasculogenesis in human ESCC too. However, in cell line secreting endogenous PEDF such as HEC46, additional PEDF gene transfer may not display the inhibitory efficacies to angiogenesis and subsequent antitumor properties though PEDF have been keeping the ability to suppress the vasculogenesis. In ESCC too, PEDF is expected to be applied to cancer therapy for antitumor effect by its potent antiangiogenic property. PEDF may have potential to exhibit the antitumor effects if tumor cells would not secrete PEDF endogenously and PEDF gene transfer would be expected to become tailor made therapy in future if patients who conform to the gene therapy, namely, who does not secrete endogenous PEDF in tumor tissue, would be ascertained.

【Conclusion】PEDF could exhibit the potent inhibitory effects to human ESCC by suppressing both angiogenesis and vasculogenesis, especially to the tumor cells lacking endogenous PEDF. Although the investigation of PEDF gene expression in tumor cells is needed for the selection of patients who receive benefit of PEDF gene therapy, PEDF gene therapy may provide a new strategy for treatment of ESCC.