称号及び氏名	博士（獣医学）　Bondoc Alexandra Ioana
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論　文　名	Characterization of Rat Amelanotic Melanoma-Derived Homotransplantable Tumor Line and Cultured Cell Lines, with Particular Reference to Tumor Microenvironments （ラットの無色素性黒色腫の同系可移植腫瘍株と培養細胞株の特性、特に腫瘍微小環境について）
論文審査委員	主査　山手　丈至 副査　渡来　仁 副査　嶋田　照雅

論文要旨

Introduction

Cancer represents a major healthcare burden worldwide with increasing frequency, being one of the leading causes of death among human population. The melanoma is a skin tumor which arises from melanocytes, generating ontogenetically in the neural crest and has a high tendency to invade and subsequently metastasize to distant sites, resulting in high mortality rate. Amelanotic melanoma is a form of melanoma in which pigmentation does not occur, and accounts for approximately 5% of all melanomas; generally, amelanotic melanomas are greater in malignant potential than pigmented melanomas.

Tumor tissues consist of neoplastic and non-neoplastic cells. The non-neoplastic cells may play important roles in regression or promotion of tumor development, by forming microenvironments evoked by intercellular communication. The non-neoplastic cells involve cancer-associated fibroblasts (CAFs), immune cells, such as tumor-associated myeloid cells (TAMCs) and lymphocytes, and vascular cells. The tumor microenvironments formed by these cells remain to be investigated in terms of melanoma tumorigenesis.

For the studies on melanoma pathogenesis, there is a constant need for animal models reproducing this neoplasm with the surrounding microenvironment. Syngeneic

tumor models are well known for a better reproducibility of tumor microenvironments, since the host immune system is not compromised. In this thesis, a new model of amelanotic melanoma was established in syngeneic F344 rats, and then, the pathobiological properties were characterized; further, the author focused on tumor microenvironments in subsequent studies. The data obtained in these studies would provide important information for melanoma pathobiology.

Chapter 1: Establishment and characterization of a homotransplantable tumor line (RMM) and cultured cell line (RMM-C) from an amelanotic melanoma in the F344 rat, with particular reference to galectin-3 expression *in vivo* and *in vitro*

Section I: A homotransplantable RMM line and RMM-C line in the F344 rat

The original tumor was found in the pinna of an aged F344 male rat. RMM was established by subcutaneous transplantation with a tumor tissue fragment into syngeneic F344 rats, with 100% intake in serial passages. Immunohistochemically, the neoplastic cells were positive to PNL-2 (melanocytes), nestin (neuroectodermal stem cells) and S-100 (neurogenic cells). Further, RMM-C was induced from an RMM tumor. RMM-C cells and the induced tumors in syngeneic rats showed immunohistochemical reactions similar to the original and RMM tumors. RMM and RMM-C are the first tumor lines of amelanotic melanoma in the rat, and may become useful tools for further studies on melanoma pathobiology.

Section II: Galectin-3 expression *in vivo* and *in vitro* in the RMM model

Galectin-3 is a galactoside binding protein with roles in tumor development. The serum levels of galectin-3 were significantly increased with RMM tumor growth. Moreover, galectin-3 expression in RMM-C cells was increased by TNF-α and decreased by TGF-β1. The obtained findings indicated that the serum galectin-3 expression level becomes a possible biomarker for melanoma development, and that the expression may be influenced by tumor microenvironments evoked by TNF-α and TGF-β1.

Chapter 2: Establishment of rat amelanotic melanoma-derived cloned cell lines and the relationship between neoplastic cells and cancer-associated fibroblast-like (CAF) cells

Section I: Neoplastic (RMM-A1) and non-neoplastic (RMM-CAF) cloned cell lines derived from the parent melanoma cell line RMM-C

Two cloned cell lines were obtained by the limiting dilution technique, from the RMM-C parent cell line from a rat amelanotic melanoma. RMM-A1 cell line was tumorigenic, with immunocytochemical positive reactions to vimentin, galectin-3 and nestin. On the other side, the RMM-CAF cell line was non-tumorigenic, showing positive reactions for α-SMA (myofibroblasts) and Thy-1 (immature mesenchymal cells). RMM-CAF was regarded as CAFs in RMM tumor tissues.

Section II: Relationship between neoplastic RMM-A1 cells and non-neoplastic RMM-CAF cells in melanoma tumorigenesis

RMM-CAF cells were co-cultured with RMM-A1 neoplastic cells. The real-time RT-PCR showed that the levels of pro-tumorigenic cytokines (TGF-β1, M-CSF, Galectin-3), chemokines (CCL-5, CXCL-1, CXCL-2), and tumor invasiveness-related factors (MMP-2, MMP-9, TIMP-1, TIMP-2) were significantly up-regulated in co-cultured RMM-CAF cells. Moreover, the neoplastic RMM-A1 cells revealed a significantly higher migration ratio in the presence of RMM-CAF cells. These results indicated that the association of CAFs with neoplastic cells play important roles in melanoma progression, indicating importance of the tumor microenvironments.

Chapter 3: Participation of tumor-associated myeloid cells (TAMCs) in the progression of amelanotic melanoma tumor line (RMM)

Section I: Macrophages and MHC class II-presenting cells in RMM tumor progression

Macrophages, including MHC class II^+ antigen-presenting cells, may play central roles in immunity against tumors. Although the total number of macrophages remained unchanged, the MHC class II^+ cells were reduced in number with tumor growth. Interestingly, further, the distribution of MHC class II^+ cells was distinguished by MHC class II^+ cell-rich areas (MHC II-HIGH) and MHC class II^+ cell-poor areas (MHC II-LOW). These results indicated that MHC class II^+ cells should play important roles in RMM tumorigenesis. However, the significance of MHC II-HIGH and MHC II-LOW areas remains to be investigated.

Section II: Functional properties of MHC class II-expressing cells in RMM model

The real-time RT-PCR after micro-dissection of MHC II-HIGH and MHC II-LOW areas, as well as fluorescence-activated cell sorting, showed that MHC class II^+ antigen-presenting cells were polarized towards M1 (high levels of IFN-γ, GM-CSF, IL-12a, iNOS), while $CD163^+$ macrophages were towards M2 (high levels of IL-10, M-CSF, Galectin-3, MMPs, TIMPs). These results indicated the importance of MHC class II and CD163 molecule expression in the polarization of TAMCs for RMM melanoma progression.

Chapter 4: Angiogenesis and the participation of tumor-associated myeloid cells (TAMCs) in RMM melanoma model

Blood vessels stainable by RECA-1 and CD34, as well as mRNA expression of pro-angiogenic factors (VEGFa and Ang-1) were increased in RMM tumors, with tumor growth. TAMCs showed implications in RMM angiogenesis; MHC class II^+ cells were localized in the proximity of blood vessels in RMM tissues, and presented the upregulated mRNA expression of pro-angiogenic factors, VEGFR-1 and Tie-2. The $CD163^+$, $CD80^+$ and $CD11c^+$ TAMCs may be implicated in tumor angiogenesis as well, through upregulation of angiogenesis-promoting factors, such as VEGFR-2 ($CD163^+$ TAMCs and $CD80^+$ TAMCs), and Ang-1 ($CD11c^+$ TAMCs).

Conclusions

The pathobiological characteristics of amelanotic melanoma were investigated by establishing rat amelanotic melanoma lines (RMM and its derived cultured cell lines).

1. RMM and its cell lines (RMM-C, RMM-A1 and RMM-CAF) are the first tools of syngeneic melanomas in rats.

2. Galectin-3 may become a possible serum biomarker of amelanotic melanoma, and its expression is influenced by tumor microenvironment factors (increase to TNF-α and decrease to TGF-β1).

3. Co-culture studies showed that the tumor-promoting cytokines and tumor invasiveness-related factors were upregulated in RMM-CAF (a cancer-associated fibroblast (CAF)-like cell line) in the presence of neoplastic RMM-A1 cell line; the cell migration of RMM-A1 cells was increased in the presence of RMM-CAF. The relationship between neoplastic and non-neoplastic cells plays important roles in melanoma progression, as a part of tumor microenvironment.

4. Tumor-associated myeloid cells (TAMCs), such as MHC class II^+ antigen-presenting cells and $CD163^+$ macrophages, had important roles in RMM tumor development: MHC class II^+ cells were predominantly polarized towards M1 type and $CD163^+$ cells were towards M2-like polarization.

5. Angiogenesis in the progression of RMM tumors was correlated with tumor growth. TAMCs showed implications in tumor angiogenesis; MHC class II^+ cells showed unique features, such as localization around the blood vessels in RMM tumors, and upregulation of angiogenesis-promoting factors. The $CD163^+$ TAMCs, $CD80^+$ TAMCs and $CD11c^+$ TAMCs may be implicated in tumor angiogenesis as well.

6. RMM and its derived cultured cell lines (RMM-C, RMM-A1 and RMM-CAF) established herein would become beneficial tools to investigate the tumorigenesis of amelanotic melanoma in relation to the tumor microenvironment, leading to possible insights underlying effective therapeutic strategies against this malignancy.

審査結果の要旨

癌患者数は世界的に増加しつつあり、国内では高齢化とともに癌は死因の第一位となっている。皮膚に生じる黒色腫は、個体発生学的には神経堤から生じるメラニン産生細胞に由来する。この腫瘍は、周囲組織に浸潤し、かつ遠隔転移し易く、死亡率の高い悪性の腫瘍として知られている。黒色腫の腫瘍細胞は、メラニン色素を含有することが特徴であるが、一方メラニン色素を有さない無色素性黒色腫がある。無色素性黒色腫は、黒色腫の約5%を占め、ヒトでは悪性度が極めて高いとされる。腫瘍組織は、腫瘍細胞と非腫瘍性細胞から成り、腫瘍細胞の増殖・転移などの生物学的挙動は、非腫瘍性細胞が作りだす微小環境に依存するとされる。非腫瘍性細胞には、間質に存在する癌関連線維芽細胞（CAF）、マクロファージなどの腫瘍関連骨髄性細胞（TAMC）、さらに血管新生に係る内皮細胞がある。黒色腫における、このような非腫瘍性細胞の特性は、ほとんど研究されていない。動物腫瘍モデルは、腫瘍の組織発生や抗癌剤の薬効を追究する上で有用である。

この一連の研究では、F344ラットに自然発生した無色素性黒色腫から、同系ラットにおける移植腫瘍株を確立し、さらに、その移植腫瘍株から培養細胞株を誘導することで、悪性黒色腫の細胞特性を、微小環境を構成する非腫瘍性細胞との係わりで解析し、黒色腫の生物学的性状に関する新たな知見を提示している。

第１章では、老齢F344ラットに自然発生した無色素性黒色腫から継代可能な皮下移植腫瘍株（RMM）と培養細胞株（RMM-C）を確立し、その細胞特性を詳細に解析している。RMMの移植率とRMM-Cの腫瘍原性はどちらも100%であり、免疫組織化学的に、これらの腫瘍細胞は、メラニン色素産生細胞や神経堤由来腫瘍に特異的なPNL-2、NestinやS-100蛋白質に対して陽性反応を示した。また、電顕観察で前メラニン顆粒が確認された。さらに、この章では、ヒトの黒色腫と同様に、Galection-3が腫瘍細胞から産生され、担腫瘍個体の血清Galection-3が腫瘍の増殖とともに増加することを明らかにしている。以上の成果は、これらの移植株と培養株が、ヒトの同等腫瘍の研究において有用であること、また、Galection-3は黒色腫のバイオマーカーになり得る可能性を提示している。

第2章では、黒色腫由来の培養細胞株RMM-Cから二つの異なるクローン細胞株（RMM-A1、RMM-CAF）を誘導し、その細胞特性を詳細に検討している。RMM-A1は、免疫組織化学的にメラニン色素産生細胞のマーカー抗原を発現し、かつ異常なクロモゾーム数を有し、同系ラットにおいて腫瘍原性を示した。一方、RMM-CAFは、CAFの特性であるα-SMAやThy-1を発現し、腫瘍原性がなかった。そこで、こ

の章では、腫瘍性細胞株であるRMM-A1と非腫瘍性細胞株RMM-CAFを共培養することで、CAFの微小環境因子としての役割を解析している。その結果、CAFから腫瘍細胞の増殖や浸潤に係るTGF-β1やCCLなどの因子が産生されることを見出している。黒色腫の悪性化に、腫瘍細胞の刺激を受けたCAFが重要な役割を演じることを提示している。

第3章では、無色素性黒色腫RMMにおける腫瘍関連骨髄性細胞TAMCの役割を腫瘍の増殖との係わりで解析している。TAMCには、腫瘍増殖に対して抑制的に働くM1マクロファージと、促進的に機能するM2マクロファージが含まれる。解析の結果、MHCクラスII発現のマクロファージは腫瘍の周辺部に特異的に存在し、機能的にはM1マクロファージに、一方、散在性にみられるCD163発現マクロファージはM2マクロファージに分極化していることを示した。黒色腫の進展に関与するTAMCの機能的特性を微小環境との係わりで明らかにしている。

第4章では、無色素性黒色腫RMMにおける新生血管の特性を解析している。その結果、RMM腫瘍の増殖に伴いCD34を発現する新生血管が増加し、さらに、TAMC、特にCD163/CD80発現のM2マクロファージは、VEGFR-2を発現することで、新生血管の誘導に係ることを明らかにしている。TAMCにより誘導される新生血管が黒色腫の悪性化に深く係ることを提示している。

本研究では、ラットの無色素性黒色腫から移植腫瘍株や培養細胞株を確立し、それらがヒトの同等腫瘍の特性を解明する上で有用なツールになり得ることを示している。さらに、非腫瘍性細胞（CAF、マクロファージ、新生血管）により作り出される微小環境が黒色腫の生物学的挙動に深く係ることを明らかにしている。この研究成果は、獣医学・医学、特に腫瘍病理学の研究分野の新たな展開に資するものであり、最終試験の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。