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学 位 論 文 題 名

Cytogenetic Abnormalities of Tumor-Associated Endothelial Cells in Human Malignant Tumors

(ヒト悪性腫瘍における腫瘍内血管内皮細胞の染色体異常)

学位論文内容の要旨

【Background and Objectives】 Tumor angiogenesis is necessary for solid tumor progression and metastasis. An important concept in tumor angiogenesis has been that tumor blood vessels contain genetically normal and stable endothelial cells (ECs), unlike tumor cells, which typically display genetic instability. However, tumor vessels and tumor-associated ECs (TECs) differ from their normal counterparts in many respects. Tumor vessels have different structural characteristics, such as fewer pericytes, leakiness, and uneven thickness of the basement membrane. Furthermore, some studies have reported that TECs possess molecular characteristics distinct from those of normal ECs (NECs). In addition, ECs derived from human renal cell carcinomas (RCCs) express biological features that are different from those of NECs. It has been reported that ECs from hematopoietic tumors harbor chromosomal aberrations. In these tumors, TECs may transdifferentiate from hematopoietic tumor cells. We have reported that ECs in nonhematopoietic malignant tumors (melanoma and liposarcoma) are cytogenetically abnormal. In mouse xenograft models, fluorescence *in situ* hybridization (FISH) analysis shows that freshly isolated mouse TECs (mTECs) are aneuploid and have abnormal multiple centrosomes. Our previous study showed that mTECs, unlike ECs in lymphomas with hematopoietic origin, did not transdifferentiate to or fuse with tumor cells because there were no human chromosomes from tumor cells in the mTEC nuclei. However, it remains to be elucidated whether these cytogenetic aberrations in mTECs isolated from malignant tumors are relevant to human TECs (hTECs) from human epithelial malignant tumors.

In the present study, we investigated chromosomal aberration in hTECs freshly isolated from RCCs (spontaneous human tumors) by FISH analysis. To study the mechanism of TEC aneuploidy, we analyzed cell-cell fusion and the relationship between progenitor marker-positive cells and TEC aneuploidy in cross-species tumor models.

【Materials and Methods】 (1). Tissues from 20 cases of renal tumor clinically diagnosed as RCCs were resected surgically. One portion of RCC or normal kidney tissue was immediately snap-frozen for immunohistology and fluorescence in situ hybridization (FISH) analysis on tissue sections. Another portion of RCC or normal kidney tissue was immediately processed to isolate ECs by magnetic cell sorting. hTECs were freshly isolated from 20 RCC tissue. hNECs were also isolated from 13 normal kidney tissue, apart from the tumor in the same specimens. FISH analysis was performed to investigate their cytogenetic abnormalities. The degree of aneuploidy was analyzed quantitatively by using chromosome 7 and chromosome 8 DNA probes in isolated hTECs or hNECs. (2). The mechanisms of TEC aneuploidy were studied using mTECs isolated from xenografts of human epithelial tumors. mTECs were isolated from human epithelial tumor xenografts grown in nude mice. OSRC-2-ECs and HSC-3-ECs were isolated from renal clear cell carcinoma (OSRC-2) and oral squamous cell carcinoma (HSC-3) xenografts, respectively. mNECs (skin ECs) were isolated from mouse dermal tissue as a control. To determine whether cell-cell fusion occurred between aneuploid mTECs and human tumor cells, dual-color FISH analysis was performed, using mouse probes and human probe. (3). To analyze the involvement of endothelial progenitor cells

(EPCs) in mTECs *in vivo*, we investigated the percentage of progenitor marker-positive cells among freshly isolated mTECs and its correlation with aneuploidy. Uncultured mTECs or mNECs were immunostained with CD133. CD133+ and CD133− mTECs / mNECs were compared for aneuploidy using quantitative FISH analysis. (4). It has been reported that centromere-associated protein-E (CENP-E) is related to aneuploidy. Thus, levels of CENP-E mRNA were analyzed in TECs and NECs by quantitative RT-PCR.

【Results】 (1). FISH analysis of immunostained tissue sections confirmed the presence of $CD31^+$ aneuploid cells, determined as aneuploid ECs, in tumor vessels. In contrast, there were no aneuploid ECs in normal renal vessels. hTECs isolated from human RCC show aneuploidy. In human RCCs, 22–58% (median 33%) of uncultured hTECs were aneuploid, whereas normal ECs were diploid. (2). mTECs isolated from epithelial tumor xenografts are aneuploid. Quantitative analysis indicated that 55% of OSRC-2-ECs and 36% of HSC-3-ECs were aneuploid. When the cells were cultured, FISH analysis showed that the frequency of mTEC aneuploidy increased to 96% and 55%. In dual FISH study, no human FISH signal in mTECs was detected (3). The percentage of CD133+ cells in mTECs was 26% (OSRC-2-ECs) and 31% (HSC-3-ECs), whereas that in mNECs was 16%. Aneupliud cells were detected in CD133+ and CD133− mTECs, whereas CD133+ and CD133− mNECs were diploid. Among mTECs, aneuploid cells were observed more frequently in CD31+CD133+cells than in CD3+CD133−cells. (4). CENP-E expression was significantly lower in mTECs (both OSRC-2-ECs and HSC-3-ECs) compared to mNECs. Furthermore, compared to all human NECs (hNECs, HMVECs, and HUVECs), hTECs expressed significantly lower levels of CENP-E.

【Discussion】 We previously reported aneuploidy in mTECs isolated from nonepithelial tumors, liposarcoma, and melanoma. However, chromosomal aberrations of hTECs isolated from human malignant epithelial tumors have not been reported.

Here we show that hTECs, like tumor cells, are cytogenetically abnormal. In order to evaluate the ploidy of hTECs quantitatively, we used hTECs freshly isolated from human RCC tissues. FISH analysis of freshly isolated and cytospun ECs provided additional evidence of hTEC aneuploidy in human RCC tissue sections. For comparative analysis, we also isolated hNECs from locations in the same specimens, apart from the tumor, during total nephrectomy. hTECs showed aneuploidy (average 33% with chromosome 7 probes and 35% with chromosome 8 probes) in all 20 samples, whereas hNECs were diploid with the 3-4% score which is in normal range and is considered to be background.

However, the mechanisms underlying TEC aneuploidy in malignant epithelial tumors are not yet understood. We addressed the mechanisms of TEC aneuploidy using cross-species tumor models that allow host cells to be distinguished from tumor cells. In the mouse epithelial tumor xenograft model, more than 35% of mTECs were aneuploid, even when not cultured. This result is consistent with our previously reported demonstrations of aneuploidy in mTECs isolated from nonepithelial tumors, liposarcoma, and melanoma. ECs in various types of tumors, epithelial or nonepithelial, may be aneuploid, suggesting chromosomal instability of mTECs. This is contrary to the traditional concept that TECs are normal and genetically stable. Our observations suggest that aneuploidy in TECs is not a rare phenomenon that is seen only in hematopoietic tumors but is actually a common feature of most malignant tumors. In our uncultured mTECs, fusion between mTECs and human tumor cells was not seen because aneuploid mTECs were not found to hybridize with human probe in the dual-probe FISH analysis.

Interestingly, $CD31^+CD133^+$ cells showed more aneuploidy compared to $CD31^+CD133^-$ cells. On the other hand, among uncultured mNECs, $CD31^+CD133^+$ cells did not include aneuploid cells. We speculate that immature ECs in tumors may have escaped cell cycle arrest and maintained aneuploidy in the tumor microenvironment. Progenitor cells with cell cycle deficiency harbor aneuploidy, and as the cells mature, their checkpoint efficiency increases. Our data demonstrating frequent aneuploidy in $CD31^+CD133^+$ cells support the hypothesis that progenitor cells contribute to mTECs aneuploidy.

Regardless of the actual mechanisms involved, our results showing the presence of chromosomal aberrations in hTECs indicate another aspect of abnormality of tumor stromal cells in carcinoma.

【Conclusion】 This is the first report showing cytogenetic abnormality of hTECs in carcinoma,

contrary to traditional belief. Aneuploid TECs that organize tumor tissue surrounding the stroma might affect tumor progression and metastasis. It is suggested that cytogenetic alterations in tumor vessels of carcinoma can occur and may play a significant role in modifying tumor-stromal interactions.

学位論文審査の要旨

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学位論文題名

Cytogenetic Abnormalities of Tumor-Associated Endothelial Cells in Human Malignant Tumors

（ヒト悪性腫瘍における腫瘍内血管内皮細胞の染色体異常）

がん組織はがん細胞だけではなく、繊維芽細胞、血管内皮細胞、血液系細胞などの間質細胞から構成されている。がん細胞は細胞生物学的に異常細胞であるのに対し、間質細胞である血管内皮細胞は正常細胞であると考えられてきた。マウスにおいてヒト腫瘍細胞のxenograftから分離された腫瘍血管内皮細胞(TEC)に染色体異常があることは報告されていたものの、ヒトがん組織中の血管内皮細胞にも染色体異常があるかは不明であった。

申請者はCD31抗体を用いて20例の腎がん検体からヒト腫瘍血管内皮細胞(hTEC)だけを分離し、hTECにFISHを行い細胞のploidyを検討した。その結果hTECはaneuploidである事が示された。また分離されたhTECを長期培養し、その性質を検討したところ、継代後のhTECでも血管内皮マーカーの発現を維持していること、またaneuploidyが増加しており核が巨大化していること、細胞分裂期のmotor proteinであるCENP-Eの発現が低下していることが示され、ヒトがん組織中の血管内皮細胞にはcytogeneticな異常があることを明らかとした。

さらに申請者はヒトがん細胞のxenograftマウスからマウス腫瘍血管内皮細胞(mTEC)を分離し、これを用いてそのTECのaneuploidyのメカニズムについて検討している。その結果、この細胞異常はがん細胞と血管内皮細胞との融合によってもたらされたものではないこと、mTECの中には幹細胞マーカーであるCD133陽性細胞が存在し、この細胞群にaneuploidyが高いことが示された。

副査の近藤　哲教授から3つの質問があった。①hTECのaneuploidyの割合が35%程度であるが、がん細胞の方のaneuploidの割合はどの程度であったのか？　②hTECのCENP-Eの発現が低下しているのはがん細胞が何らかの因子を出している為なのか？　③hTECがaneuploidyを伴うような異常細胞であることは、がんにとってみれば、増殖に有利と考えられるのか？

申請者は上記質問に対し①CD31陰性の分画に存在する腎がん細胞はhTECよりaneuploidyが高く、腎癌細胞株でのFISHでも細胞のほとんどはaneuploidであること　②正常血管内皮細胞(NEC)をがん細胞のconditioned mediumで暴露すると細胞異常が起こるという実験結果を例に出し、がん細胞がリリースする未知の因子があり、その中にhTECのCENP-Eの発現を低下させる因子も含まれている可能性があること　③aneuploidyのmTECは薬剤抵抗性があり、増殖因子に対する反応性が高い事実があり、TECにaneuploidyがあることはがんの増殖にとって有利だと推測する　と回答した。

主査の笠原　正典教授から6つの質問があった。④hTECの染色体異常の詳細はどうであ

ったのか？　⑤全染色体のセットが倍加するような変化であるのか？　⑥数の異常だけではなく構造異常はあったのか？　⑦腎がんだけでなく他のがん種でも腫瘍血管内皮細胞のaneuploidyがあるのか？　⑧腎癌のsubtypeによりhTECのaneuploidyの割合に相違は見られるのか？　⑨CD133陽性のmTECにaneuploidyが高い理由はなぜか？

申請者は上記質問に対し④karyotypeまでは調べていないため詳細は分からないが、がん細胞と同じようなランダムな異常であると推測されること　⑤マウスでの過去の報告ではkaryotypeが調べられており、各染色体がランダム増減していたこと、⑥マウスの報告では数の異常だけではなく転座などの構造異常もみとめられたことから、hTECでも同様に構造異常もあるのではないかと推測されること　⑦pilot studyで肺がん、肝臓がん、食道がんでも腫瘍血管内皮細胞のaneuploidyは見られること　⑧subtypeとaneuploidyの割合に相関はないこと　⑨幹細胞などimmatureな細胞ではcell cycle arrestが働きにくくなっているとの報告があり、結果としてaneuploidの細胞であってもapoptosisに至らないためではないか　と回答した。

副査の野々村　克也教授から４つの質問があった。⑩他のがん種から分離されたTECと本実験での腎がんから分離されたTECとの間に相違はあるのか？　⑪腎がんでは血管新生阻害を目的とした分子標的薬が臨床適応されているが、TECで同薬剤の感受性は高いのか、低いのか？　⑫抗がん剤に対する薬剤感受性はどうか？　⑬骨髄由来の血管内皮細胞はがん組織ではどこに存在しているのか？

申請者は上記質問に対し⑩比較していないためわからないが、腎がんはhypervascularであり分離される血管内皮細胞の数も多いため、がん細胞からの影響も多くなるのではないかと推測すること　⑪分子標的薬に対する薬剤感受性は調べていないが、TECはNECと比較し感受性が低いと推察されること、⑫マウスのTECでは5-FUおよびvincristineなどの抗がん剤の感受性が低いとの報告があること、⑬抗がん剤治療後のがん組織において中心がnecrosisとなって退縮しても周辺部は増殖していることがあるが、この際骨髄由来の血管内皮細胞は増殖部分に集積しているとの報告があり、骨髄由来の血管内皮細胞はがんの増殖が活発な部分に存在するのではないかと推測している　と回答した。

この論文は，ヒトがん組織でもTECのaneuploidyが存在することを初めて提示した点で高く評価され，今後のがん組織におけるがん微小環境を解明する発端になると期待される。審査員一同は、これらの成果を高く評価し、大学院課程における研鑽や取得単位なども併せ申請者が博士（医学）の学位を受けるのに充分な資格を有するものと判定した。