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HISTOLOGICAL BASIS OF MR/OPTICAL IMAGING OF HUMAN MELANOMA MOUSE XENOGRAPTS SPANNING A RANGE OF METASTATIC POTENTIALS

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Abstract

Predicting tumor aggressiveness will greatly facilitate cancer treatment. We have previously reported investigations utilizing various MR/optical imaging methods to differentiate human melanoma mouse xenografts spanning a range of metastatic potentials. The purpose of this study was to explore the histological basis of the previously reported imaging findings. We obtained the cryogenic tumor sections of three types of human melanoma mouse xenografts with their metastatic potentials falling in the rank order A375P<A375M<C8161. Both H&E and DAPI counter-stained TUNEL analysis showed distinct core-rim difference in aggressive tumors, while the core has apparently many viable cells forming structure of vascular-like networks and the rim appears viable-cell dense. The least aggressive ones (A375P) are relatively more homogenous without distinct core-rim difference. However, our previous study showed the core of more aggressive melanoma has higher Fp/NADH redox ratio, indicative of nutritional deprivation. Additionally, the low perfusion/blood vessel permeability measured previously by DCE-MRI indicated these cells should be under starvation presumably accompanied with more cell death. Thus, it remains an open question what the survival status of the cells in the core of more aggressive melanoma is. We are currently investigating whether these cells are in autophagic state, a possible cell survival mechanism under starvation conditions.

1. INTRODUCTION

Predicting tumor metastatic potentials has high clinical significance since aggressive tumors in general exhibit faster growth progression and higher metastasis than less aggressive ones. Our long term goal is to identify imaging markers of metastatic potentials (or aggressiveness) in mouse models of human melanoma and eventually translate these markers into clinical applications. We have established mouse xenografts of five melanoma cell lines (A375P, A375M, A375P5, A375P10, and C8161), whose metastatic potentials in mouse models and/or invasive potentials *in vitro* have been investigated.^{1–6} We have reported in previous ISOTT meetings that DCE-MRI and T₁ρ-MRI can differentiate the most aggressive melanoma xenografts from the least aggressive ones,⁷ and that low temperature

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NADH/Fp fluorescence imaging or redox scanning can distinguish the aggressiveness of five melanoma cell lines (reported at ISOTT 2006 Conference). The results from the three methods all showed distinct difference between tumor core and rim in more aggressive melanomas. For example, DCE-MRI indicated that the aggressive C8161 tumors have a well-perfused rim and poorly-perfused core; $T_{1\rho}$ -weighted MRI gave smaller $T_{1\rho}$ value in the areas near the necrotic center of the aggressive C8161 tumors; the redox scanning results also showed the least aggressive tumor A375P is largely reduced with low redox ratios and the other four types of more aggressive tumors have oxidized core with high redox ratios and reduced rim with low redox ratios. The mean redox ratios in the more oxidized core highly correlate with the invasive potentials of the five lines measured in Boyden chamber ($R^2=0.93$). This seems to suggest that it is the core that accounts for the differences in the metastatic potential. As part of our research efforts to understand the results obtained from the MR/optical imaging methods, we further performed histological analysis of frozen tumor sections. In this paper, we report our preliminary research findings and some insights obtained from H&E and DAPI counter-stained TUNEL assay analysis of three types of human melanoma mouse xenografts with their metastatic potentials falling in the rank order A375P<A375M <C8161.

2. METHODS

2.1. Animal Models

Three human melanoma cell lines, A375P, A375M, and C8161 were cultured in 10% FBS-added RPMI 1640 medium at 37°C under 5% CO₂. The metastatic potentials of A375P, A375M, C8161 have been evaluated by counting the number of metastasis in distant organs in mice inoculated with human melanoma cell lines,¹⁻³ with C8161 being the most aggressive and A375P being the least aggressive. The invasive potentials of the three cell lines were also measured by the *in vitro* Membrane Invasion Culture System (MICS),^{5,6} falling in the rank order A375P(3%)<A375M(7%) <C8161(13.5%). The propagated cells were collected when they reach 75% confluence and subcutaneously inoculated into the athymic nude mice and grew into tumors in about 4–8 weeks. Two size groups of tumor were chosen for our study: smaller than 7 mm and bigger than 10 mm in diameter. In total, we have studied 13 tumors.

2.2. Histological Analysis of Tumor Tissue Sections by H&E and DAPI Counter-stained TUNEL Assay

The tumors were excised and immediately placed in OCT medium and stored at - 20°C. The OCT-embedded frozen-tissue samples were then sliced 10µm thick at various depths of the tumor, with the slice plane in parallel with the mouse body surface. The frozen sections were stained by H&E and DAPI Counter-stained TUNEL Assay for histological examination. Adjacent sections were used for both H&E and TUNEL analysis, respectively.

H&E-stained tumor section slides were examined under microscope and photo images were taken with a bright light microscope at 40X. In addition, several dozens of images were photo-stitched using Photoshop® software to obtain the image of a whole tumor section for each type of tumor.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science) as per the manufacturer's instructions. Cryo-preserved melanoma tissue sections treated with DNase (DNase I recombinant, Grade I) prior to the TdT reaction served as the positive control. Sections processed in the absence of TdT served as the negative control. All sections were counter-stained with DAPI contained in the mounting medium (Vectashield®) and protected by cover-slip and edge-sealed with nail polish. The TUNEL assayed slides were stored in the dark at 4°C before and after images were taken. Fluorescent photo images were acquired with a NIKON 600 upright microscope equipped with a digital camera. In addition, several dozens of adjacent images of both DAPI and FITC were photo-stitched using Photoshop® software to obtain the image of a whole tumor section for each type of tumor.

3. RESULTS AND DISCUSSION

3.1. H&E stained sections

The more aggressive melanomas showed high histological heterogeneity in terms of the distinct core and rim difference in both size groups. Distinct histological difference from core and rim of the more aggressive A375M and C8161 tumors was readily seen with the rim being more bluish and the core more reddish as shown in Figure 1(a). In addition, the histology of the core appears more like fishnet constituted of viable cells. Similar structure is also often observed in the metastatic melanoma patients and is called vascular-like network⁸ that is rich in extracellular matrix.^{9, 10} Most of the cells were morphologically different from the necrotic ones but also different from the ones in the rim. Their intact nuclei indicate that they were not completely dead yet. The rim was viable-cell dense.

No distinct difference from core and rim was observed from the least aggressive A375P tumors (Figure 1(b)) although very few small areas of morphologically different cells can be found sporadically in the bigger size group, indicating the histological homogeneity relative to the more aggressive ones.

3.2. DAPI counter-stained TUNEL assay results:

Correlating well with the histological result from H&E, results from TUNEL assay, in terms of core and rim difference, also showed high heterogeneity for the more aggressive A375M and C8161 melanomas (Figure 1(c) & (g)); while the least aggressive A375P tumors did not have core and rim difference (Figure 1(e)).

Vascular-like networks in the core of the more aggressive tumors can be seen readily. DAPI images clearly show many intact nuclei constituting the histological networks regardless whether the tumors appear to have visible ulceration or not (note: all tissue sections were carefully sliced to have excluded any ulceration). This is consistent with the observations from the H&E analysis. The FITC images give more interesting results. For the bigger aggressive tumors, very few fluorophore colocalizations in the overlay image of DAPI and FITC images were found, suggesting very few TUNEL positive cells. The large area having bright fluorescence that shows no color colocalization with DAPI staining in the FITC

images of the more aggressive tumors is presumably background of autofluorescence. For the smaller aggressive tumors with visible ulceration, relatively more TUNEL positive cells were found in the tissue section near the ulcerated area compared to the bigger ones, indicating some of the core cells were apoptotic. The rim of the more aggressive tumors was viable-cell dense and no apoptotic cells were found regardless of the tumor size.

Also consistent with the H&E result, the least aggressive A375P tumors were viable-cell dense, showing no distinct core and rim difference. The bigger one has <10% of apoptotic cells in sporadic areas. Almost no TUNEL-positive cells were found in the smaller ones.

3.3. Discussion

Our previous DCE-MRI study indicated limited blood/nutrient supply in C8161 tumor core, which suggests the core of the aggressive melanoma should be accompanied by increased cell death. We also observed previously that the core of more aggressive melanoma has higher Fp/NADH redox ratio. High Fp/NADH ratios have been shown to indicate high oxidative metabolism in mitochondria.^{11, 12} High Fp/NADH ratios were also seen in heart tissues under ischemia, indicating that cells were under starvation and cell death was occurring.¹³ Hence, high Fp/NADH ratios indicate hypermetabolic activity or starvation/cell death. However, in our histological study, we found very few apoptotic cells in the core of the bigger aggressive ones and low percentage of apoptotic cells in the core of the smaller aggressive ones. Meanwhile we observed a large number of intact nuclei from DAPI images of the core. DAPI (4'-6-Diamidino-2-phenylindole) is known to form fluorescent complexes with natural double-stranded DNA. The intactness of the nuclei seen in our DAPI images suggests these cells were still viable. From the corresponding H&E images, we observed that these cells maintained their integrity although they were morphologically different from the ones in the rim, which also suggests high likelihood of their viable status. Thus, it remains an open question what the survival status of the cells in the core of more aggressive melanoma is.

Cancer cells may undergo apoptosis when under metabolic stress or treatment. However, study shows that the apoptosis-defective cells evade immediate death fate by entering into autophagic state.¹⁴ An autophagic cell lives by eating its own intracellular macromolecules, or eating neighboring cells (cannibalism).^{15, 16} Autophagy can be a cell-survival mechanism,^{17, 18} and may be linked with poor treatment prognosis in melanoma clinic (personal communication with Ravi Amaravadi at the Department of Medicine, University of Pennsylvania).

Our prior experience indicates that melanoma growth rates seem to positively correlate with their invasive potentials. For example, the tumor volumes measured at day 33 (N=3~4 for each cell line) correlate with the invasive potentials of the 5 cell lines with a correlation coefficient of $R^2=0.9662$ (unpublished data). Whether the imaging/histological findings reflect the difference in tumor invasiveness or growth rate or both is still a question for future investigation.

4. CLOSING REMARKS

We have reported our histological findings from H&E staining and TUNEL assay on frozen tissue sections of human melanoma xenografts in athymic nude mice. In summary, for more aggressive melanomas, we found 1) tumor core and rim is distinctly different; 2) vascular-like networks constituted by viable cells dominate the core; 3) bigger tumors have very few apoptotic cells; 4) the core of the smaller tumors without visible necrotic center have more apoptotic cells than that of the bigger ones; 5) the smaller tumors with necrotic center still have many viable cells in the core; 6) tumor rim is viable-cell dense. For the least aggressive melanomas, we found that they appear to be relatively more homogenous, viable-cell dense. No distinct core-rim difference was observed. For bigger tumors, the less aggressive ones seem to have more apoptotic cells than the aggressive. This was not the case for the smaller ones. We may need to study more tumors to verify our preliminary results. It may be useful to have more quantitative measurements of the amount of apoptosis and necrosis in different types of tumors.

Since our previous study indicates that the tumor core seems to hold the answer for tumor aggressiveness, and in our preliminary histological study we did not find many apoptotic cells in the core but rather viable cells that are morphologically different from those in the rim were present, it is necessary to understand their survival status. We are currently investigating whether these cells are in autophagic state, a possible cell survival mechanism under starvation conditions.

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Figure 1. (a)-(h) Photo-stitched images of a tumor section and (i)-(j) expanded view of a core area of the most aggressive C8161 showing few apoptotic cells (20X). (a) & (b) H&E staining image (40X) of tumor sections of A375M (a) and A375P (b); FITC (c) and DAPI (d) of A375M and FITC (e) and DAPI (f) of A375P; FITC (g) and DAPI (h) of C8161; (i) FITC of C8161, and (j) DAPI of C8161. The diameter of the tumors shown here is greater than 11 mm. The strong fluorescence in Figure 1(c) is not specific FITC-signal for apoptosis, rather it maybe tissue autofluorescence.