Review

Viewing Malignant Melanoma Cells as Macrophage-Tumor Hybrids

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tumor cell fusion, tumor macrophage hybrid, melanoma, metastasis, melanin, migration, adhesion, β 1,6-branched oligosaccharides

ABBREVIATIONS

CMM	cutaneous malignant melanoma
LAMP	lysosome associated membrane protein
TYRP	tyrosinase related protein
GnT V	β 1,6 N acetylglucosaminyl-transferase V

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ABSTRACT

Cutaneous malignant melanoma (CMM) begins in the epidermis as the clonal emergence of melanocytes having a deregulated mitotic cycle. In a manner not yet understood, some descendants of these cells loosen their adhesions in situ and migrate into the dermis, thus initiating the processes of invasion and metastasis. These cells look and act much like macrophage-melanoma hybrids created in the lab or arising in mice. But genetic proof for hybrids in human melanoma is still lacking. Nonetheless, should tumor cell hybridization account for the invasive phenotype, this would surely evoke new therapeutic approaches regarding mechanisms of cell fusion and hybrid-specific molecular signatures. Here are described some of the remarkable phenotypic similarities between experimental macrophage-melanoma hybrids and CMM. The results suggest that invasive and metastatic CMM might well arise through fusion and genomic hybridization between melanoma cells and migratory bone marrow-derived cells.

INTRODUCTION

Most solid cancers begin in the epithelium as carcinomas or melanomas. If detected while still confined to their site of origin, they can be removed and the patient be cured. But outcome becomes less and less favorable as the cells migrate through the basal lamina into the dermis, intravasate into blood vessels and lymphatics, and disseminate to nearby lymph nodes, distant tissues and organs. These complex feats require the cancer cell to drastically change its phenotype: modifying adhesions, activating extracellular matrix proteinases and turning on the machinery for chemotactic motility. To accomplish this, cancer cells notoriously acquire phenotypes of migratory bone marrow-derived cells such as macrophages.^{1,2}

In the prevailing view, acquisition of the migratory phenotype results from genetic instability and random emergence of cells with new gene expression patterns. Some of these cells develop capabilities for migration and are compatible with host selective pressures in foreign environments such as the dermis and circulatory system or in hypoxic regions. By this view, the metastatic cell arises progressively and step-wise as advantageous genes for metastasis are activated or amplified and disadvantageous genes silenced or lost. Related concepts involve the activation of master genes for multiple molecular pathways such as those involved in epidermal-mesenchymal transition (EMT) during development.³

In an alternate view, the emergence of new gene expression patterns in cancer is less a step-wise process but a consequence of fusion of cancer cells with migratory bone marrow-derived cells.^{1,2,4,5} In such fusion hybrids, the array of expressed genes, the epigenome, reflects the outcome of mixing imprinted genomes from the different developmental lineages. Some of the hybrids express the deregulated cell division of the cancer cell along with the migratory capabilities of the bone marrow cell, giving them increased potential for invasion and metastasis. While this is decidedly not a prevailing view of tumor progression, tumor cell fusion has been demonstrated in many different systems. Tumor cells fuse with a variety of normal cell types including cells of the endothelium,⁶ stroma,⁷ and bone marrow-derived cells.⁸⁻¹¹ In vivo fusions between bone marrow-derived cells and human renal carcinoma cells,^{8,9} colon carcinoma cells,¹⁰ and between myeloma cells and osteoclasts¹¹ have been reported. Of the more than 30 studies of tumor cell fusion in vivo, none has failed to detect hybrids. Many implicate macrophages or other bone marrow-derived cells as fusion partners. And many further implicate hybridization as the cause of metastasis.^{1,2}

Figure 1. Macrophage-melanoma hybrids are hypermelanotic. Left, Non-pigmented Cloudman S91 melanoma, the parental fusion partner. Right, Highly melanized melanoma-macrophage hybrid 48.³⁵

A vast repertoire of molecules and traits is shared by macrophages and melanoma cells. Some are associated with angiogenesis, matrix alterations, motility, chemotaxis and immune signaling pathways. Others involve expression of macrophage-like phenotypes or are immunomarkers used in the identification of macrophages.^{1,2} Macrophage-tumor cell fusion could explain the aneuploidy, plasticity, and heterogeneity of malignant melanoma and it could also account for epidermal-mesenchymal transition in tumor progression since macrophages are of mesodermal origin.^{1,2}

This article focuses on the possibility of metastatic melanoma occurring as an outcome of fusion between melanoma cells and migratory bone marrow-derived cells such as macrophages. Proof for the spontaneous appearance of melanoma hybrids has been obtained in mice via molecular genetics and karyotype analyses. However there is as yet no genetic ‘smoking gun’ in human melanoma and the arguments below for hybrids in CMM are based on phenotypic similarities to known melanoma hybrids in mice and in vitro models.

MELANOMA HYBRIDS ARE HYPERMELANOTIC, CONTAIN COARSE MELANIN AND EXHIBIT β 1,6-BRANCHED OLIGOSACCHARIDES

Three reports describe melanoma-host hybrids arising spontaneously in mice: one of B16 melanoma cells¹² and two of Cloudman S91 melanoma cells.^{13,14} In all three cases, the hybrids were

hyperplod compared to the parental melanoma cells and, curiously, were hypermelanotic, producing excessive amounts of melanin. Most important, these lines showed markedly higher tumorigenicity¹² and metastatic potential.¹³⁻¹⁵ We analyzed two of these hybrids and found that they had acquired the capacity for chemotactic migration in vitro and unexpectedly produced ‘coarse melanin’, autophagosomal vesicles with multiple melanosomes (below). Likewise, when mouse or human macrophages were experimentally fused with Cloudman S91 melanoma cells in vitro, more than half of the 75 individual hybrids showed this same phenotype.^{15,16} Highly metastatic, hypermelanotic macrophage-melanoma hybrid 48 in culture below (Fig. 1, right) compared to the non-pigmented parental Cloudman S91 melanoma cells (Fig. 1, left). The melanized regions of hybrid 48 are denoted with arrows.

EM analyses of hybrid 48 demonstrated that the cells produced autophagic coarse melanin. Shown in Figure 2 (left panel) is an electron micrograph of a glutaraldehyde-fixed specimen of a hybrid 48 tumor growing in a dba/2J mouse. The cell contains melanosome-laden autophagosomes in various stages of the digestive pathway, including dense, smaller vacuoles of autophagosomes fused with lysosomes (autophagolysosomes).¹⁷

The observation that macrophage-melanoma hybrids are autophagic has assumed new importance since recent studies show that autophagy is widespread in cancer and represents a dominant tumor cell survival mechanism.¹⁸⁻²¹ It is also a characteristic of macrophages.^{22,23} Evidence below suggests how this may in part result from a macrophage glycosylation system being superimposed on a melanocytic phenotype.

β 1,6-BRANCHED OLIGOSACCHARIDES REGULATE MOTILITY AND MELANOGENESIS

The hypermelanotic, autophagic phenotype was puzzling. Why should fusion of nonpigmented macrophages with weakly-pigmented melanoma cells yield highly pigmented hybrids? The short answer is aberrant glycosylation in the form of β 1,6-branched oligosaccharides. Analyses of melanosomal proteins tyrosinase, TYRP-1, TYRP-2, and LAMP-1 in macrophage-melanoma hybrids showed that they were heavily glycosylated compared to parental melanoma cells.²⁴ That LAMP-1 was one of these proteins provided the first indication that β 1,6-branched oligosaccharides might be involved. The lysosome-associated membrane proteins (LAMP’s 1, 2 and 3) are some of the most heavily glycosylated of all proteins and are

Figure 2. Left, EM of melanoma-macrophage hybrid 48. Right, EM of a B16F10 mouse melanoma cell. Both cells were within tumors implanted in mice. Each cell line produced coarse melanin: autophagosomes and autophagolysosomes containing multiple melanosomes and other cytoplasmic structures. The autophagosomes are in various stages of the autodegradative pathway from the early stage vesicles “aph” to smaller and denser vacuoles generated by fusion with lysosomes (nuc, nucleus; aph, autophagosome).

Figure 3. β 1,6-branched oligosaccharides colocalize to coarse melanin in macrophage-melanoma hybrid 48. Left: Coarse melanin autophagosomes in fixed, unstained cells after growth in culture. Right: The same cells after bleaching to decolorize melanin and staining for β 1,6-branched oligosaccharides with the lectin LPHA. Arrows denote colocalization between individual melanin-containing vesicles and β 1,6-branched oligosaccharides.³⁵

chief substrates for GnT-V, a glycosyltransferase that is rate-limiting in the formation of β 1,6-branched oligosaccharides.²⁵⁻²⁷ GnT-V has the characteristics of a master regulator of metastasis. It acts on multiple glycoprotein substrates, particularly those involved in cell adhesion and motility, and thereby activates pathways in metastatic progression.²⁸⁻³¹ High GnT-V expression is a macrophage trait and this along with the glycosylation of LAMP-1 indicated that GnT-V was likely to be elevated in macrophage-melanoma hybrids. This was subsequently verified through histochemistry, flow cytometry, and direct measurements of GnT-V mRNA.³²

Using the lectin LPHA (leucocytic phytohemagglutinin) and histochemistry to detect β 1,6-branched oligosaccharides, LPHA stained several parts of the cell, including the plasma membranes, but staining was particularly strong in coarse melanin autophagosomes (Fig. 3). Subsequent studies revealed that both the metastatic and melanogenic phenotypes in hybrids were regulated by GnT-V and β 1,6-branched oligosaccharides.³¹ This was deduced from experiments in which GnT-V was inhibited following transfection of GnT-III, a competitive inhibitor of GnT-V. β 1,6-branched oligosaccharides were markedly lowered, with concomitant decreases in both chemotactic motility and melanin production.³¹ Moreover,

it is possible that GnT-V itself was an inducer of autophagy and the formation of coarse vesicles. Harari et al demonstrated that GnT-V transfection into mink lung cells induced production of LPHA- and CD63/LAMP-3-positive multilamellar vesicles whose formation was dependent on autophagy.³³ This suggested that GnT-V expression might have induced autophagy. Thus in principle, by adding β 1,6-branched oligosaccharides to melanogenic proteins and by stimulating autophagy, GnT-V could have elicited both increased melanogenesis and the packaging of melanosomes into autophagosomes. By glycosylating proteins involved in adhesion and migration, GnT-V could have elicited a metastatic phenotype. It is shown below that human malignant melanoma cells also exhibit high GnT-V activity and share similar phenotypes with experimental macrophage-melanoma hybrids in mice.

HISTOLOGICAL CORRELATES IN MOUSE AND HUMAN MELANOMA

Prompted by the above findings, we looked for similar phenotypes in established melanoma cell lines and in pathology specimens of CMM.

B16F10 mouse melanoma. From the vast number of citations, the B16 melanoma is surely the most widely studied of all the melanoma models. In light of the discussion herein, it is important to note that in retrospect the *in vivo* generation of B16F10 melanoma cells was favorable for host-tumor hybrid formation.³⁴ B16 tumor cells were passaged repeatedly from culture, to mice, to culture. Each time, lung nodules were dissociated, placed in culture and “colonies with melanin granules” were again passaged in mice.³⁴ As mentioned above, there are three reports that *in vivo* passage of melanoma cells produced host-melanoma hybrids.¹²⁻¹⁴ B16F10 melanoma cells expressed the same phenotype as these hybrids: high metastatic potential, chemotactic motility, hyperpigmentation, high expression of GnT-V and β 1,6-branched oligosaccharides, and autophagic coarse melanin (Fig. 2, right panel). Thus, the possibility is raised that B16F10 melanoma cells originated as macrophage-tumor hybrids. However, since B16F10 melanoma cells were generated in syngeneic (C57B6) mice, it has not been possible to determine this on a genetic basis.

SKMel-23 melanoma cells. A similar phenotype of autophagic coarse melanin positive for β 1,6-branched oligosaccharides and CD63/LAMP-3 was also observed in SKMel-23 human metastatic melanoma cells.³⁵ Pathology specimens of human CMM. A major diagnostic feature of primary CMM is heterogeneous pigmentation

Figure 4. Human cutaneous malignant melanoma in situ. A. Coarse melanin autophagosomes in an H&E-stained section (arrows). B. A sequential section bleached to decolorize melanin and stained for β 1,6-branched oligosaccharides with the lectin LPHA. C. A sequential section bleached and stained with anti-CD63/LAMP-3. Tumor-associated melanophages are denoted “m”. (In C, while melanophages are present they do not stain with CD63/LAMP-3.)^{28,36}

Figure 5. (A–C) Melanophages (arrows) staining blue-green with azure blue in close association with melanoma cells staining brown with the S100-immunoperoxidase reaction. Images are from archival pathology cases of human CMM.³⁶

with prominent hyper- and/or hypomelanotic regions. Studying archival pathology specimens of CMM we found that melanoma cells in the hypermelanotic regions of CMM produce coarse melanin.^{28,36} As in macrophage-melanoma hybrids above, coarse melanin in CMM was shown through EM studies to consist of autophagosomes and autophagolysosomes filled with melanosomes and other cytoplasmic elements.^{37–41} Moreover, coarse melanin in CMM was strongly positive for β 1,6-branched oligosaccharides and CD63/LAMP-3.^{28,36} Shown in Figure 4 are pathology tissue sections from a highly melanized case of CMM in situ. Figure 4A shows an H&E-stained section with arrows denoting coarse melanin. Figure 4B and C show sequential sections stained with LPHA and anti-CD63/LAMP-3 respectively with arrows denoting staining of coarse melanin. In contrast, healthy melanocytes peripheral to tumors were far less pigmented and produced neither coarse melanin nor β 1,6-branched oligosaccharides.²⁸

The staining patterns in Figure 4 were common in CMM.^{28,36} In melanoma tissue microarrays with more than 500 primary and metastatic tumors, >80% of the tumors were positive for β 1,6-branched oligosaccharides and coarse vesicles.³⁶ Similar patterns were common in all 22 types of nonmelanoma cancers examined, except for the absence of melanin.²⁸ Thus, this phenotype is shared by macrophage-melanoma fusion hybrids, melanomas, and a variety of human cancers in addition to melanoma.

Other metastasis-associated genes highly expressed by macrophages, macrophage-melanoma hybrids, and CMM are cMet proto-oncogene (encodes receptor for hepatocyte growth factor/scatter factor),⁴² SPARC (secreted protein acidic and rich in cysteine; osteonectin; BM40)⁴³ and MCR1 (melanocortin-1 receptor; melanocyte stimulating hormone receptor).⁴⁴ (From the phenotypes and activated genes described, it is also likely that melanocyte master regulator gene MITF was activated, but these measurements were not performed). Each of these stimulates melanoma motility in vitro. While the above correlations do not prove that expression of these genes in human cancer is a consequence of tumor-macrophage fusion, they are certainly consistent with the possibility.

MELANOPHAGES AND MELANOMA CELLS ARE CLOSELY ASSOCIATED IN VIVO

The melanoma tumor environment is rich in melanophages (macrophages that ingest melanin). Macrophages actively phagocytose apoptotic cells. Oncogenes in apoptotic cells can be horizontally transferred via phagocytosis where they confer a tumorigenic phenotype.⁴⁵ Macrophages also express inherent mechanisms for

cell-cell fusion as in the formation of osteoclasts and multinucleated giant cells.⁴⁶ Figure 5 shows pathology specimens of human melanomas stained for melanophages (arrows) by the S100/azure blue technique. Melanophages containing numerous large vesicles are seen in close association with melanoma cells with long segments of plasma membranes of the two cell types in apposition to one another (Fig. 1A and B), or with melanophages engulfing melanoma cells (Fig. 1C). Physical contact such as this would be a prerequisite for fusion and macrophage-tumor hybrid formation. Thus, it would seem that the melanoma tumor environment is conducive to macrophage-melanoma fusion.

CONCLUSIONS AND FUTURE DIRECTIONS

Metastatic CMM is notoriously recalcitrant to treatment. Understanding the mechanisms through which melanoma cells are able to loosen adhesions in situ and migrate away from the primary tumor is of great importance. It is here proposed that human malignant melanoma is a disease of tumor-macrophage hybridization. Similar proposals have been presented earlier.^{4,15,47,48} Although there certainly are other views for melanoma progression, it would seem they need to take into account the remarkably similar phenotypes shared by human melanomas in vivo and experimental melanoma-macrophage hybrids, especially the widespread upregulation of GnT-V activity.

In a word, malignant melanoma cells look and act just like macrophage-melanoma hybrids created in the lab or arising in mice. But genetic proof for hybrids in human melanoma is still lacking. Cases in which patients develop melanoma following allogeneic bone marrow transplant could provide such evidence through detection of bone marrow donor genes in host tumor cells. Should it be determined that melanoma fusion is a significant mechanism for tumor progression, new treatment strategies would surely follow. The early processes of fusion and genomic hybridization would seem to present new and vulnerable targets for therapeutic intervention.

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