Fibronectin-Containing Extracellular Vesicles Protect Melanocytes against Ultraviolet Radiation-Induced Cytotoxicity

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Skin melanocytes are activated by exposure to UV radiation to secrete melanin-containing melanosomes to protect the skin from UV-induced damage. Despite the continuous renewal of the epidermis, the turnover rate of melanocytes is very slow, and they survive for long periods. However, the mechanisms underlying the survival of melanocytes exposed to UV radiation are not known. Here, we investigated the role of melanocyte-derived extracellular vesicles in melanocyte survival. Network analysis of the melanocyte extracellular vesicle proteome identified the extracellular matrix component fibronectin at a central node, and the release of fibronectin-containing extracellular vesicles was increased after exposure of melanocytes to UVB radiation. Using an anti-fibronectin neutralizing antibody and specific inhibitors of extracellular vesicle secretion, we demonstrated that extracellular vesicles enriched in fibronectin were involved in melanocyte survival after UVB radiation. Furthermore, we observed that in the hyperpigmented lesions of patients with melasma, the extracellular space around melanocytes contained more fibronectin compared with normal skin, suggesting that fibronectin is involved in maintaining melanocytes in pathological conditions. Collectively, our findings suggest that melanocytes secrete fibronectin-containing extracellular vesicles to increase their survival after UVB radiation. These data provide important insight into how constantly stimulated melanocytes can be maintained in pathological conditions such as melasma.

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INTRODUCTION

Extracellular vesicles (EVs) are nanosized phospholipid-bilayer spherical particles that are secreted by most cells to facilitate intercellular communication (Bang and Thum, 2012; Simons and Raposo, 2009). Transcriptomic and proteomic profiling has revealed that EVs contain enzymes, receptors, structural and extracellular matrix proteins, transcription factors, and nucleic acids that perform diverse functions in recipient cells (Guescini et al., 2010; Kim et al., 2013a, 2014; Valadi et al., 2007). For example, EVs derived from stem cells are involved in tissue repair (Lai et al., 2010), whereas those derived from dendritic cells are important for antigen presentation and immune responses (Thery et al., 2002). Exosome-like vesicles derived from *Plasmodium falciparum*-infected erythrocytes facilitate communication within parasite populations (Regev-Rudzki et al., 2013), and tumor-derived EVs play a critical role in oncogenesis (Lima et al., 2011; Saleem and Abdel-Mageed, 2015) and favor the establishment of melanoma metastasis, possibly by upregulating TGF-β1 production and consequently down-modulating macrophage activation (Lima et al., 2009). A recent study also demonstrated that EVs derived from highly metastatic melanomas promoted the metastatic potential of primary tumors by signaling to bone marrow progenitors via the transfer of hepatocyte growth factor receptors (Peinado et al., 2012).

Fibronectin (FN) is a major extracellular matrix component that is involved in cell proliferation, migration, differentiation, and survival (Geiger et al., 2001; Kosmehl et al., 1996). FN is an EV protein (Deng et al., 2012; Kim et al., 2013a; Yu et al., 2006), as confirmed by multiple proteomic analyses (Kim et al., 2013a). The FN-containing EVs (FN-EVs) released from breast tumor cells in tumor tissue, where leukocytes participate in the induction of FN-EVs from tumor cells, promote tumor cell invasion and metastasis via activation of the focal adhesion kinase/Src-dependent signaling pathways (Deng et al., 2012). In EVs derived from trophoblasts and ovarian cancer cells, FN is a key molecule that induces the production of the proinflammatory cytokine IL-1β in macrophages (Atay et al., 2011). Furthermore, EVs from MDA-MB-231 breast cancer and U87 glioma cells confer the transformation phenotype to normal fibroblasts and epithelial cells by transferring tissue transglutaminase and FN, which induce signaling events that enhance cell survival and aberrant growth (Antonyak et al., 2011).

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Abbreviations: ACN, acetonitrile; CD81, cluster of differentiation 81; EV, extracellular vesicle; FN, fibronectin; PBS, phosphate buffered saline

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Melanocytes located in the basal layer of the skin epidermis secrete and transfer melanin-containing vesicles called melanosomes to neighboring keratinocytes to protect keratinocytes and the skin underneath them from UV radiation-induced damage (Hearing, 1993). Basal keratinocytes are highly proliferative and undergo a well-defined differentiation program that includes an apoptotic process, resulting in the continuous renewal of the epidermis. Unlike keratinocytes, melanocytes display low proliferative potential and are highly resistant to cell death because of their constitutive expression of the anti-apoptotic protein Bcl2, which leads to their sustained survival in the epidermis for decades (Rinnerthaler et al., 2014). Because of these characteristics, melanocytes are vulnerable to mutations that can occur over time as a result of repetitive UV exposure, which can cause hyperpigmentation disorders or the formation of melanomas. Given that melanocyte survival is pivotal for maintaining epidermal homeostasis because they prevent UV-induced DNA photodamage and tumorigenesis (Bertolotto, 2013; Luciani et al., 2011), it is critical for melanocytes to maintain their genomic stability, and other protection mechanism(s), besides BCL2, seem to be necessary. In this study, we examined the effect of melanocyte-derived EVs on the survival of melanocytes that were exposed to UVB radiation. We found that UVB enhanced the secretion of EVs that contained FN, which promoted the survival of UVB-irradiated melanocytes. Our findings provide important insight into melanocyte intercellular cooperation during protection against UVB-induced damage and the promotion of their survival under stressful conditions.

RESULTS

Human primary melanocytes release FN-EVs

Dynamic light scattering analysis of EVs from the cultured supernatants of human primary melanocytes revealed that they were homogeneous in size, with an average diameter of 195.10 ± 17.40 (Figure 1a). Proteomic analysis identified 262 proteins (Supplementary Table S1 online), 73% of which were included in the EVpedia (Kim et al., 2013a, 2014), compared with the 12% of EV proteins in the whole human proteome (UniProt). Among these proteins, EV-enriched proteins such as cluster of differentiation 81 (CD81) and heat shock protein 90 were identified, and their expression was further confirmed by western blot analysis (Figure 1b), wherein they showed enrichment in only the EV fraction and not in the medium control. Of the 262 proteins identified, 174 (66.4%) were annotated with at least one Gene Ontology cellular component term, and the composition of proteins related to the cell surface, lipid rafts, and the extracellular matrix (approximately 67.8%) was relatively high when compared with the composition of those in whole proteome (Figure 1c, Supplementary Table S2 online), implying the possible involvement of EVs in intercellular interactions and communication. The network analysis of the identified EV proteins, performed using the STRING database (version 9.05) (Franceschini et al., 2013) and the network-visualizing program Cytoscape (version 2.8.3) (Shannon et al., 2003), revealed a network of 39 proteins (Figure 1d). FN1 occupied a critical position in this network, that is, it had the highest centrality based on degree, betweenness, closeness, and radiality (Supplementary Table S1) among membrane proteins (blue nodes, Figure 1d). We next examined whether melanocyte-derived EVs contained FN by western blot analysis. To verify the FN signal, melanocytes were treated with forskolin, which is known to induce FN production (Dean et al., 1989). EVs isolated from mock-treated melanocytes contained FN, and the level of FN was increased by forskolin treatment (Figure 1e), suggesting that melanocytes inherently release FN-EVs. Confocal microscopy analysis demonstrated that FN colocalized with the EV marker CD81 on vesicular structures inside or on melanocyte dendrites (Figure 1f).

Fibronectin in extracellular vesicles is increased by UVB radiation

Because FN is secreted to promote cell survival, we investigated whether FN-EV secretion can be regulated. We treated melanocytes with UVB radiation, a major stressor of skin cells, and analyzed the release of EVs by western blot analysis using anti-EV marker (CD81 or heat shock protein 90) antibodies and determined the FN content in the EVs either by silver staining of SDS-PAGE gels or by western blot analysis. We found that the levels of CD81 and heat shock protein 90 were increased in the EVs isolated from UVB-treated melanocytes (Figure 2a, second and third panels) and that FN levels were increased in EVs (Figure 2a and b, first panels) but decreased in cell lysates (Figure 2b, second panel) without changes in mRNA expression (Figure 2c). Because the average sizes of the EVs isolated from UVB-treated or untreated melanocytes were similar (Figure 1a, Supplementary Figure S1 online), these data imply that UVB radiation may accelerate not only EV secretion but also the loading of FN from the intracellular pool into EVs. To confirm that FN is loaded into and secreted with EVs, we treated melanocytes with two different EV biogenesis inhibitors: the SMase inhibitor GW4869, which is the general inhibitor of EV secretion, or the p53 inhibitor pifithrin-μ, which inhibits EV secretion through a p53-dependent pathway (Yu et al., 2006), before UVB exposure. Both inhibitors decreased FN levels in EVs and levels of the EV marker CD81 (Figure 2d and e) but increased FN levels in cell lysates (Figure 2f and g), indicating that FN is loaded into and secreted with EVs after UVB radiation, probably through a p53-dependent pathway.

In the human skin epidermis, a single melanocyte is surrounded by 20–40 keratinocytes (Hoath and Leahy, 2003), and UVB radiation could also affect FN-EV secretion by these keratinocytes, which would contribute to the EV pool around the melanocytes in the skin. Therefore, we examined the release of FN-EVs by keratinocytes after UVB radiation. However, our results indicated that UVB did not stimulate FN-EV secretion by keratinocytes; moreover, the amount of FN in keratinocyte-derived EVs appeared to be different from the amount in melanocyte-derived EVs (Figure 3a). We used human dermal fibroblasts, which secrete FN to the basement membrane (Dean et al., 1989), to confirm FN signal and to investigate UV responsiveness. Similar to melanocytes, fibroblasts released EVs containing FN, as indicated by a single band above 98 kDa that was increased by UVA radiation, a major stressor of dermal fibroblasts (Pohl and Christophers, 1979) (Figure 3b). Our data indicate that in

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