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Original Research Article

Fibroblasts isolated from the malignant melanoma influence phenotype of normal human keratinocytes

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CAF, cancer associated fibroblast
DF, dermal fibroblasts
K, high molecular weight keratins
K14, keratin type 14
MAF, melanoma-associated fibroblast
Vim, vimentin

ABSTRACT

Intercellular interactions are able to influence the biological properties of many types of tumors including malignant melanoma. Differentiation pattern of melanoma cells is significantly influenced by the melanoma-associated fibroblasts but the information about interaction of these cells with other important element of melanoma microenvironment, resp. with keratinocytes, is limited. In this, study we tested the effect of fibroblasts isolated from malignant melanoma on phenotype of normal human keratinocytes, especially on their expression of vimentin, a cytoskeletal protein weakly expressed in normal human keratinocytes. The co-culture with normal dermal fibroblasts was used for comparison. The results demonstrated the high expression of vimentin in keratinocytes co-cultured with melanoma-associated fibroblasts compared with those co-cultured with normal dermal fibroblasts. These data suggest participation of melanoma-associated fibroblasts-keratinocyte crosstalk in formation of melanoma niche.

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Introduction

Malignant melanoma is highly aggressive tumor with increasing incidence and very restricted possibilities of tumor treatment in an advanced stage of the disease (Forsea et al., 2012). Melanoma cells lack their aggressiveness when they are implemented to early embryo (Kulesa et al., 2006). Moreover, the melanoma-associated fibroblasts (MAF) seem to be able to influence biological properties of melanoma cells including their phenotype (Comito et al., 2012; Kodet et al., 2013). Melanoma cells, similarly to their precursors neural crest cells originated from the hair follicle, are able to significantly influence normal keratinocytes *in vitro* as well as *in vivo* (Kodet et al., 2015). It indicates that the melanoma microenvironment, similarly to other types of tumors, where the microenvironment is important for control of biological properties of cancer cells, is influenced mainly by its cell components. Several types of cells such as inflammatory elements, cancer-associated fibroblasts (CAF) and endothelial cells were discovered as elements influencing biological properties of cancer cells (Plzák et al., 2010; Smetana et al., 2013). Unfortunately, only the very limited number of data is available about the MAF-keratinocytes interaction in regulatory cascades of melanoma and their role in tumor biology. However, some role of these cells on keratinocytes can be expected because CAF are able to significantly influence phenotype of normal keratinocytes (Kolář et al., 2012). To evaluate the possible role of MAF on keratinocytes, we isolated fibroblasts from skin metastasis of advanced skin melanoma and co-cultured them with normal human skin interfollicular keratinocytes. The data were compared with co-culture of normal human breast dermal fibroblasts (DF) and breast keratinocytes. We visualized keratin 14 as a marker of basal proliferating cells and co-expression of keratins and vimentin as a marker of epithelial–mesenchymal transition.

Materials and methods

Cells processing

DF and normal keratinocytes were prepared from healthy residual skin of the breast of patient undergoing esthetic procedure. MAF were prepared from women with cutaneous metastases of malignant melanoma: Breslow 4.0 and Clark IV removed during palliative alleviating procedure in stage IV (TNM). The isolation of all three cell types was published (Lacina et al., 2007a; Kolář et al., 2012). All tissues used in this study were obtained and handled in agreement with the approval of the local ethical committee with respect to the Declaration of Helsinki, protection of patient's rights and benefits and strictly with informed consent of each patient. To study the effect of fibroblasts to keratinocytes, we opted for direct co-culture. MAF of the 4th passage were seeded on coverslips in the density 1000 cells/cm^2. 24 h later normal human keratinocytes of 3rd passage were added in the density 30,000 cells/cm^2 and cultured in keratinocyte medium at 37 °C and 5% CO_2 for 6 days (Strnad et al., 2010). The control experiment with normal human DF was performed by the same procedure.

Immunocytochemistry

Cells were fixed with 5% paraformaldehyde in buffered saline solution (PBS) at pH 7.3, carefully washed with PBS and permeabilized by Triton X-100 (Sigma–Aldrich, Prague, Czech Republic). Monoclonal mouse antibody against keratin type 14 (K14) (Sigma–Aldrich, Prague, Czech Republic), monoclonal mouse antibody against vimentin (Vim) (DAKO, Glostrup, Denmark) and polyclonal rabbit antibody against panel of high molecular weight keratins (K) (Abcam, Cambridge UK) were used. The FITC-labeled swine anti-mouse antibody (DAKO, Glostrup, Denmark) and TRITC-labeled goat anti-mouse antibody (Sigma–Aldrich, Prague, Czech Republic) were employed as the second-step antibodies. All antibodies were diluted as recommended by supplier. Nuclei were counter-stained by 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich, Prague, Czech Republic). The control of the specificity of reaction was performed by the irrelevant antibodies, in case of monoclonal of the same isotype to exclude the nonspecific interaction of antibodies via interaction of Fc fragment of immunoglobulin with Fc receptor. Imaging was performed using Eclipse 90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany) and the computer-assisted image analysis system LUCIA 5.1 (Laboratory Imaging, Prague, Czech Republic).

Results and discussion

Keratinocytes cultured on both types of fibroblasts i.e. MAF and DF formed distinct colonies. While the keratinocytes co-cultured with MAF were highly positive for K14 as marker of basal cells, only the keratinocytes on periphery of colonies exhibited the strong signal for this intermediate filament when they were co-cultured with DF (Fig. 1A and B). Expression of K was not affected by the type of co-cultured fibroblasts (Fig. 1C and D). This pattern of K14 highly positive cells was observed in relevance to melanoma biology in colonies of keratinocytes stimulated by melanoma cells or in periphery of keratinocyte colonies treated with FGF-2, VEGF-A, IL-8 and CXCL-1, i.e. products of melanoma/neural crest stem cells influencing the differentiation pattern of epidermal keratinocytes overlaying the nodular melanoma (Kodet et al., 2015).

Keratinocytes co-cultured with MAF were positive for presence of Vim, that was co-expressed with K, also namely on the periphery of their colonies. This phenomenon was negligible in keratinocyte colonies co-cultured with DF (Fig. 1C and D). These Vim-positive cells usually exhibited the elongated anterior–posterior polarized shape (Fig. 1C and $C_{1–3}$) that is typical for migrating cells. Moreover, the signal for Vim was also highly polarized with maximum in the leading edge-like pole of epithelial cell (Fig. 1$C_{1–3}$). We observed frequently this phenomenon in keratinocytes co-cultured with CAF from squamous cell carcinoma and with cells of benign fibrous histiocytoma (Lacina et al., 2007b; Kideryová et al., 2009). Similarly, high double positivity for K and Vim was also observed in co-culture of keratinocytes with melanoma cells and hair follicle neural crest originated stem cells (Kodet et al., 2015). Vim seems to be important for normal human

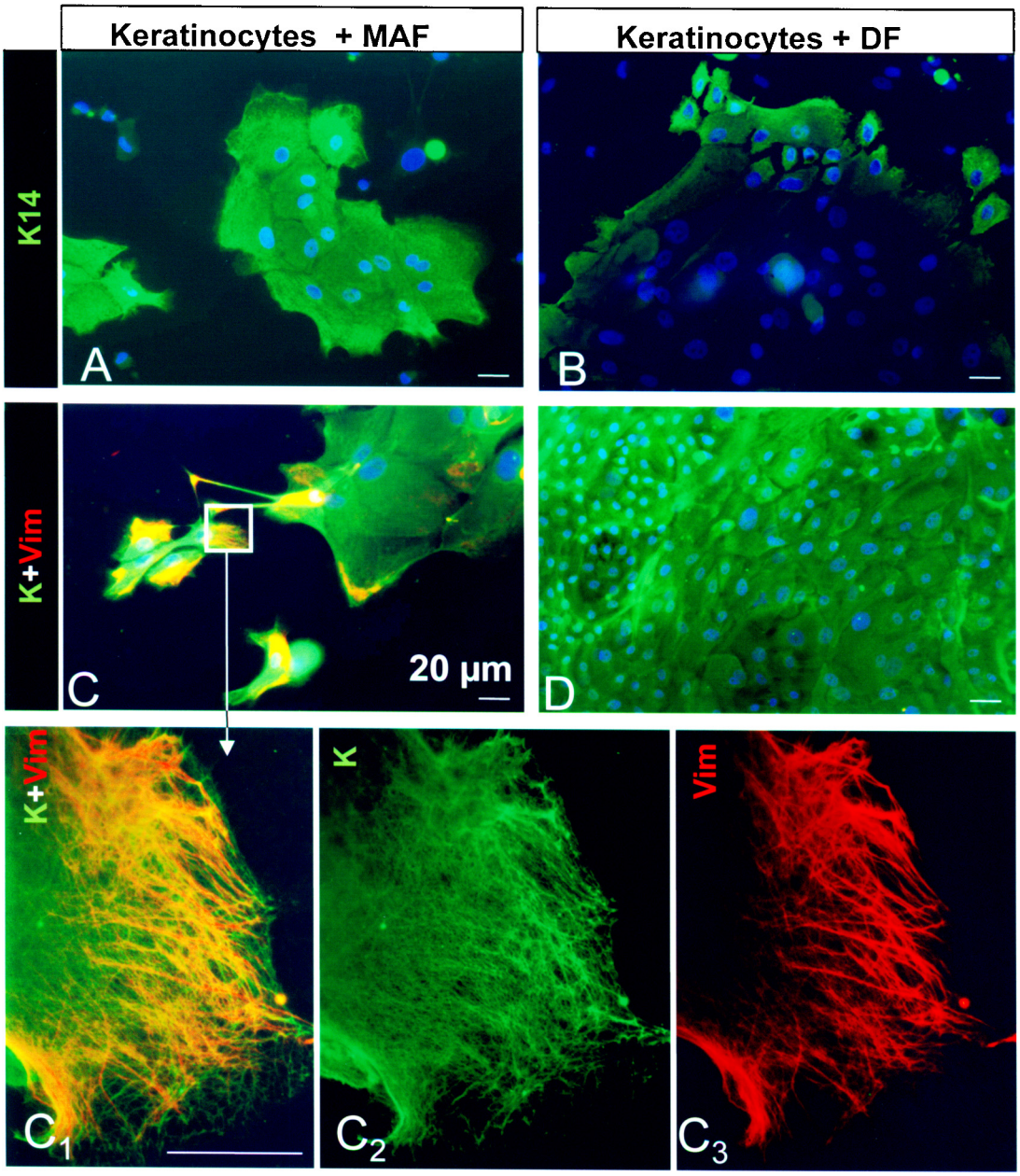

Fig. 1 – Expression of keratin 14 (K14, green signal, A, B), of panel of high molecular weight keratins (K, green signal, C, D) and vimentin (Vim, red signal, C, $C_{1–3}$, D) in keratinocytes co-cultured with melanoma-associated fibroblasts (MAF, A, C, $C_{1–3}$) and with normal dermal fibroblasts (B, D). Bar 20 μm.

keratinocytes during their extensive clonal growth (Castro-Munozledo et al., 2015). Moreover, Vim expression in keratinocytes co-cultured with CAF is also frequently accompanied by nuclear expression of transcription factor Snail that is participating in the process of epithelial–mesenchymal transition. (Lacina et al., 2007b). Data presented in this study demonstrate that MAF are able to influence not only melanoma cells (Comito et al., 2012; Kodet et al., 2013) but also keratinocytes. Similarly, melanoma cells are also able to influence keratinocytes (Haass et al., 2010; Kodet et al., 2015). Moreover, the effect of MAF seems not to be tumor type specific because these cells are also able to influence uniformly the phenotype of breast cancer cells (Dvořánková et al., 2012). The described effect of MAF on keratinocytes can be relevant from the point of view of the melanoma growth and spreading because activated keratinocytes are able influence migration of melanoma cells (Kesswell et al., 2012).

In conclusion, MAF represent an important constituent of the complex microenvironment where melanoma cells, MAF and keratinocyte with participation of immune cells cooperate and so influence the malignant melanoma progression.

Conflict of interest

The authors declare no conflicts of interest.

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