Changes in the Levels of N-Cadherin and PCNA in Skin Melanoma Cells Are Mediated through Matrix Metalloproteinase 9

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Immunohistochemical studies revealed increased level of matrix metalloproteinase 9 in skin melanoma cells. Inhibition of matrix metalloproteinase 9 with interfering RNA changed the level of PCNA and reduced N-cadherin content in melanoma cells. This attests to the involvement of matrix metalloproteinase 9 in the realization of invasion and metastatic growth and in the regulation of tumor growth and progress.

Key Words: *matrix metalloproteinase 9; skin melanoma; N-cadherin*

Matrix metalloptoteinases (MMP) are endopeptidase class enzymes involved in intracellular matrix degradation and remodeling [5]. Realization of these effects is essential for tumor cells at the stage of migration and metastatic growth. Matrix metalloproteinase 9 is synthesized as a proenzyme with a molecular weight of 92 kDa. After activation MMP-9 destroys collagen IV, denatured collagen, and basement membrane components, which is an important event in the realization of invasive growth by the tumor [6]. High levels of MMP-9 were detected in breast cancer, colorectal and gastric cancer [4]. The possibility of using MMP-9 as a marker of skin melanoma vertical growth phase has been suggested [9]. In addition to induction of degradation of intracellular matrix components, MMP-9 is involved in the regulation of the basic biological processes, apoptosis, proliferation, and differentiation [10]. However, these effects of MMP are far less studied.

We evaluated the expression of PCNA and N-cadherin (protein associated with invasive tumor growth) in melanoma cells after MMP-9 inhibition with RNA interference.

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MATERIALS AND METHODS

Immunohistochemical studies were carried out on skin melanoma biopsy specimens from 102 patients and on intact skin biopsy specimens from normal subjects.

Skin specimens were fixed in 10% neutral buffered formalin. Sections (5 μ) were routinely stained with monoclonal antibodies to MMP-9 (Abcam, 1:250). Ready-to-use detection system (Novocastra) with diaminobenzidene (Novocastra) was used for visualization. The sections were then post-stained with hematoxylin. Positively stained cells were counted under an Olympus BX-41 microscope (×400). Positively stained cells were counted for tumor cells and intact epidermis separately. The intensity of cell staining was evaluated in points: 0: no staining; 1: weak staining; 2: moderate staining, and 3: marked staining. The immunocytochemical (ICC) index was then calculated by the formula:

P(i)=% stained cells with different intensity× staining intensity score.

The results were interpreted as follows: 0-10 — negative reaction; 10-100 – slightly positive; 100-300 — positive reaction.

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TABLE 1. Comparative Analysis of MMP-9 Expression in Skin Melanoma (*M*±*m*)

Skin layer	Intact skin from healthy individual (control)	Melanoma, intact skin, ICC index	Melanoma, tumor cells, ICC index
Epidermis	5.0±0.3	78.1±6.2*	198.5±18.2*
Derma	2.10±0.05	63.5±8.1*	-

Note. Here and in Table 2: *$p<0.05$ in comparison with the control.

Melanoma cell culture SK-MEL-1 was a kind gift from A. N. Sysin Institute of Human Ecology and Environmental Hygiene, the Russian Academy of Medical Sciences. Skin melanoma cells were cultured in 12-well plates in RPMI with glutamine and BSA in a CO_2 incubator at 5% CO_2. After the concentration of 1.5×10^5 cell/ml was attained, transfection with interfering RNA (iRNA) was carried using Lipofectamine 2000 (Invitrogen). iRNA were synthesized by Sintol Company in accordance with sequences determined by E. Meyer [7]. In 24 h, the cells were fixed in 10% formalin with subsequent ICC staining by the standard protocols using monoclonal antibodies to MMP-9 (Abcam, 1:250), PCNA (1:400), and N-cadherin (1:400). Negative control was carried out without first antibodies in order to rule out nonspecific cell staining. Scrambled RNA sequences were used in a special series of experiments. Positively stained cells were counted, cell staining intensity index and ICC index were evaluated, and the results were interpreted as described previously.

The results were statistically processed by Kruskal–Wallis test for related groups using Statistica 6.0 software.

RESULTS

Matrix metalloproteinases 9 were detected in normal skin in keratinocytes and fibroblasts. In the skin of melanoma patients, MMP-9$^+$ cells were detected in tumor complexes, epidermis, and metastatic lymphocytes, but not in the primary tumor. The level of MMP-9 was 15-fold elevated in intact sites of melanoma epidermis in comparison with normal human intact skin epidermis. Its level was almost 40-fold elevated in the skin epidermis of melanoma patients in sites of tumor complexes location in comparison with the control. The expression of MMP-9 was 30-fold elevated in melanoma derma in comparison with its level in intact human skin derma (Table 1).

Evaluation of MMP-9 expression in melanoma cell culture showed ICC index of 244.25 in the control. The ICC index dropped to 100 after RNA interference and remained at 237.7 in cells incubated with scrambled RNA (Table 2). Changes in the intensity of MMP-9$^+$ cells staining after addition of interfering and scrambled RNA indicated realization of iRNA effects. In addition, we studied changes in the expression of PCNA (proliferating cell nuclear antigen), a polymerase-δ DNA cofactor. It is known that the level of PCNA increases significantly during the S phase and correlates with bromodeoxyuridine incorporation degree, this suggesting PCNA as an adequate marker of cell proliferation [1,2]. Inhibition of MMP-9 caused a virtually 3-fold decrease of PCNA expression. Suppression of β-catenin signal mechanism and disorders in adhesion contacts between the cells are probable mechanisms leading to changes in the melanoma cell level after MMP-9 blockade. It has been found, for example, that iRNA inhibition of MMP-9 in smooth muscle cells causes proliferation reduction and a decrease of nuclear β-catenin level in cells, which is known to be due to D1 cyclin and c-Myc genes negative control [3].

Inhibition of MMP-9 functional activity led to reduction of N-cadherin level. Cadherins are a family of proteins regulating cell-cell adhesion contacts. Normally epithelial cells (including melanocytes) express E-cadherin, while replacement of E-cadherin with N-cadherin on melanocyte surface is associated with the so-called epithelio-mesenchymal transition phenomenon, associated with the tumor phenotype

TABLE 2. Levels of MMP-9, PCNA, and N-Cadherin in Melanoma Cells after Incubation with iRNA to MMP-9 (*M*±*m*)

ICC index	Control	iRNA	Scrambled RNA
Count of MMP-9$^+$ cells	244.25±10.60	100.0±5.2*	227.7±8.6*
Count of PCNA$^+$ cells	280.7±12.3	100.0±3.8*	270.6±5.3
Count of N-cadherin$^+$ cells	211.5±9.4	60.2±2.4*	207.6±7.1

formation. N-Cadherin is an important regulator of tumor cell migration, which is related to manifestations of invasive properties of tumor cells. Changes in functional activity of β-catenin-signal mechanism are also the most probable cause of MMP-9-mediated modification of N-cadherin level in our experiment. It is known, for example, that β-catenin and N-cadherin cytoplasmatic domains form complexes essential for realization of adhesion contacts by N-cadherin [8].

Hence, inhibition of MMP-9 in skin melanoma cells was paralleled by reduction of tumor characteristics of cells; for example, PCNA level changed, presumably indicating reduced proliferation of the studied cells; the expression of the protein regulating cell migration and invasion also changed. These changes indicate that the role of MMP does not consist just in destruction of extracellular matrix and stimulation of neoangiogenesis, as was assumed previously. We can hypothesize that MMP-9 can serve as a potential therapeutic target in skin melanoma and modulation of functional activity of this enzyme can serve as a method for tumor growth inhibition.

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