Provisional chapter

# Expression of Matrix Metalloproteinases and Theirs Tissue Inhibitors in Fibroblast Cultures and Colo-829 and SH-4 Melanoma Cultures After Photodynamic Therapy

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Additional information is available at the end of the chapter

## 1. Introduction

Metalloproteinases (MMPs) are a numerous group of proteolytic enzymes whose activity depends on $Zn^{2+}$ and $Ca^{2+}$ ions [1-2]. They participate in various physiological and pathological processes. They are engaged in the processes of restructuring and degradation of the basement membrane and components of the extracellular matrix (ECM) [3]. So far, 25 enzymes from that group have been characterized (22 in humans), as well as 4 their inhibitors (tissue inhibitors of metalloproteinases - TIMPs) [4-5]. Gene expression of metalloproteinases occurs in almost all cells: in fibroblasts, keratinocytes, macrophages, endothelium cells, Langerhans dendritic cells, neurons, microglial cells, myocytes, as well as in inflammatory infiltration cells: in monocytes, T lymphocytes [5]. Increased activity of metalloproteinases can be observed in various pathological states, i.e. in neoplastic growths, arthritis, periodontal diseases, atherosclerosis, dilated cardiomyopathy, myocardial infarction and diseases of the skin [6]. In case of neoplasms, a possible dependence is often mentioned: quantity and activity level of these enzymes can influence advancement of the tumour. Results of many studies show importance of the expression profile of various MMPs and their inhibitors on the prognosis and course of the disease [7]. Generally, changes in proportion of particular MMPs in the whole MMP pool negatively influence the prognosis. However, in some cases, like e.g. in colorectal cancer, changes in the expression profile due to increased expression of MMP-12 and MMP-9 are connected with longer survivability of the patient and decreased ability of neoplastic cells to cause metastasis [8, 9]. Studies on the amount of inhibitors metalloproteinase matrices (TIMPs) have proven that there is a relation between a high level of TIMP-1 and -2, and an unfavourable prognosis for the patient. The

cause of an increase in concentration of TIMPs is probably higher expression of MMPs and an attempt to maintain an equilibrium between MMP and TIMP expression in a neoplastic process [7]. TIMPs may also favour neoplastic progression through inhibition of apoptosis of neoplastic cells (TIMP-1 and -2), stimulation of their growth (TIMP-2 and -3) or participation in the beginning of angiogenesis in a tumour (stimulation of VEGF secretion – TIMP-1) [10]. Moreover, TIMPs show activity independent from MMPs, e.g. they cause increased levels of anti-apoptotic protien BCL-XL [11] and have an influence similar to the growth factor [12]. Tests performed on animals also proved that TIMP-2, despite its name, is an important activator of MMP-2 [13].

### 1.1. Participation and role of MMPs in the course of a neoplastic process

There are six basic changes in the physiology of a cell which underlie neoplastic development [14]:

- self-delivery of signals that stimulate growth,
- lack of sensitivity to signals that inhibit growth,
- escape from apoptosis,
- unlimited proliferation,
- stimulation of angiogenesis,
- invasiveness and ability to create metastasis.

The studies conducted in the last few years confirmed participation of metalloproteinases in several stages of neoplastic development.

#### *1.1.1. MMPs and regulation of growth of tumour cells*

Three ways in which MMPs contribute to proliferation of tumour cells are known. Firstly, MMPs release precursors of some growth factors, such as e.g. TGF-$\alpha$ [15] that are related to the cell membrane. Secondly, MMPs may participate in the process of activation of those factors, e.g. MMP-24 and MMP-25 activate IGF factors (insulin-like growth factors) through proteolysis of IGF-BPs (insulin-like growth factor binding proteines). Thirdly, metalloproteinases influence composition of ECM and thus can indirectly regulate proliferation signals through integrins [16]. MMPs can also influence, in a negative way, growth of neoplastic cells through TGF-$\beta$ or through participation in production of proapoptotic factors such as Fas ligand (FasL) or TNF-$\alpha$ [7].

#### *1.1.2. MMPs and regulation of apoptosis*

MMPs demonstrate both apoptotic and anti-apoptotic activity. MMP-3, -7, -9 and MMP-11 are metalloproteinases with particular significance attributed to them. It was proved that overexpression of MMP-3 in epithelial cells of mammals induce apoptosis, which is probably a result of laminin degradation [17-19], while MMP-7 can release cell membrane-related FasL – transmembrane stimulator of the FasL death receptor. Released FasL can induce apoptosis of neigh-

bouring cells or cause inhibition of neoplastic cells [20-21]. MMP-7 may also inhibit apoptosis through digestion of pro-HB-EGF (inactive form of the heparin-binding epithelial growth factor) and secretion of mature HB-EGF which support cell survival [22]. Anti-apoptotic influence of MMP-11 is often described with an example of IGFs (insulin-like growth factors) release, which can be factors that increase survivability of cells [23-24]. During apoptosis of endothelium, metalloproteinases also participate in digestion of VE-cadherin (vascular endothelial cadherin) [25] and cell adhesion molecules such as: PECAM-1 (a cell adhesion molecule from the immunoglobulin superfamily) and E-cadherin (epithelial cadherin). Degradation of the aforementioned cell adhesion molecules may be a cause for typical rounding of apoptotic cells [26].

*1.1.3. MMPs and regulation of angiogenesis*

An important factor in the growth of a tumour is creation of new blood vessels within its region [14]. Metalloproteinases play an important role as positive regulators of that process. Tests conducted on animals have proven that both endogeneous and synthetic inhibitors of metalloproteinases cause decreased angiogenesis [27]. Huge significance is here attributed to the following metalloproteinases: MMP-2, -9, -14, which directly influence the course of the angiogenic process, and MMP-19, which is expressed in blood vessel cells [28]. In case of MMP-2 overexpression, a development of a mysterious feature of $\alpha v\beta$-integrin was described within type-IV collagen. Blocking that spot with antibodies inhibits migration of endothelial cells in the process of *in vitro* angiogenesis and limits growth of the tumour [29]. MMP-9 causes higher availability of pro-angiogenic VEGF factor, however the mechanism of its action has not yet been explained [30]. It is believed that also MMP-14 contributes to the angiogenesis of the tumour as antibodies aimed at the catalytic domain of MMP-14 inhibit migration of endothelial cells, as well as invasiveness and creation of capillary vessels *in vitro* [31]. MMP-14 can also degrade fibrillary matrix which surrounds newly created blood vessels, which may potentially disable endothelial cells to further penetrate the tumour tissue [32]. MMPs also contribute to creation of molecules which are inhibitors of angiogenesis. Digestion of plasminogen by MMP-2, -3, -7, -9 and -12 results in creation of angiostatin [33]. MMP-3, -9, -12, -13 and MMP-20 may also be engaged in creation of endostatin (C-terminal fragment derived from type XVIII collagen of basement membranes) [34]. Both angiostatin and endostatin reduce proliferation of endothelial cells [35]. Additionally, endostatin may inhibit invasiveness of endothelial cells, acting as MMP-14 and MMP-2 inhibitor [36]. MMP-12 may also inhibit angiogenesis of the tumour through digestion and cutting off the receptor related to the cell membrane, that is urokinase-type plasminogen activator (u-PA), which is necessary in the process of invasion of endothelial cells into a fibrin matrix [37].

*1.1.4. MMPs and invasiveness and metastasis*

During metastasis, neoplastic cells have to overcome several barriers put in their way by ECM. Firstly, they have to get through the basement membrane of the endothelium and infiltrate the surrounding stroma. Then they have to infiltrate the blood or lymphatic vessels (intravasation), migrate through vessel walls to the surrounding tissue (extravasation) and create new, proliferable metastatic foci [3].

Digestion of lamina-5 by MMP-2 and MMP-14 contributes to creation of a digestion product, which facilitates cell movements. Such phenomenon can be observed in experimental *in vivo* tumours, while in case of human neoplasms a common location for both MMP-14 and lamina-5 was found [7. However, MMP-14 may also participate in digestion of CD44 receptor (the main receptor for hyaluronic acid) which resides on the surface of neoplastic cells. That receptor has an ability to bond MMP-9 and, therefore, attributes to the location of the enzyme on the surface of the cell. Such location of MMP-9 is necessary to initiate invasion of the tumour cells and angiogenesis within its boundaries. Therefore, MMP-14 may contribute to a decrease in MMP-9 activity on the surface of the cell, which subsequently may inhibit invasive properties of the tumour cells and angiogenesis [38].

Progression of the tumour is also connected with disturbance of the functioning of the E-cadherin cell adhesion molecule, which is digested by MMP-3 and MMP-7. Secretion of an E-cadherin fragment due to a cut attributes to the invasion of neoplastic cells in the paracrine way [39]. Digestion of E-cadherin also contributes to transformation of the epithelial cells into the mesenchymal tissue [40].

During the invasion of tumour cells, placement of metalloproteinases on the cellular invadopodia is necessary for them to acquire the ability of invasiveness. MMP-2, -9 and -14 are metalloproteinases identified as those which appear within the invadopodia, however the placement mechanisms are different for each of them [41].

Metalloproteinases also participate in late stages of the metastatic process when neoplastic cells have to intrude blood and lymphatic vessels, survive in them and, finally, evacuate from them. At that stage, the experimental models describe overexpression of MMP-9 and MMP-14, which correlates with the growth in number of neoplastic cells which survive in experimental tests of metastasis [42- 43].

It is believed that there are tissue-specific differences in occurrence of proteinases which initiate metastasis. Moreover, activity of TIMPs in those tissues, which in some cases inhibit recurrence of neoplastic foci, also seems to be different [7].

### *1.1.5. MMPs and immunological response*

One of the characteristics of a neoplastic disease is occurrence of inflammatory responses [44]. The immunological system is able to recognize and attack neoplastic cells. However, those cells have an ability to "escape" supervision and control of the immunological system. Those phenomena may, to some extend, depend on metalloproteinases [7].

Inflammatory infiltration within a tumour contains cytotoxic T lymphocytes, NK cells and macrophages which target neoplastic cells. Proliferation of T lymphocytes is regulated by cytokine signals, precisely by interleukin-2 receptor-$\alpha$ (IL-2R$\alpha$) [7].

Metalloproteinases, including MMP-9, can only digest IL-2R〈and in that way inhibit proliferation of T lymphocytes [7]. Metalloproteinases are also an activator of TGF-$\beta$ [45], an important T lymphocyte response inhibitor targeted against neoplastic cells [46]. The product of digestion of 〈1-proteinase inhibitor created by MMP-11 causes decreased sensitivity of neoplastic cells

to an attack from NK cells. MMPs may also target the chemokines themselves, which results in a growth or reduction of infiltration and migration of leukocytes [ADD CITATION]. MMP-9 shows ability to digest neutrophil chemotactic factor CXCL8 (interleukin-8; IL-8), therefore making it ten times more active. Moreover, MMP-9 inactivates the CXCL7 precursor (a Type-III connective tissue activating peptide; CTAP-III), CXCL4 (platelet factor 4; PF4) and CXCL1 (growth-related oncogene-$\alpha$; GRO$\alpha$) [47].

MMP-1, -3, -9, -13, -14 can participate in digestion and inactivation of CXCL12 (stromal cell-derived factor 1, SDF-1) [48], which is a ligand for the CXCR4 receptor (SDF-1 chemokine receptor) located on the leukocytes. It is interesting that breast cancer cells also show expression of CXCR4, while inhibition of binding CXCL12 to CXCR4 through blockage with use of antibodies significantly reduces *in vivo* metastasis to lungs and lymph nodes. Therefore, digestion of CXCL12 by MMPs may inhibit metastasis [49].

Although the immunological system reacts to cancer cells and probably delays progression of the neoplastic disease, existence of a chronic inflammatory state may also influence development of tumours of the skin, breasts, prostate, ovary, mucous membrane of the stomach, large intestine, liver and bladder. In progression of neoplastic disease of animal models, inflammatory cells such as mastocytes, neutrophil granulocyte and macrophages participate in the process [44]. Those cells synthesize several types of MMPs, including -9, -12 and -14, therefore release of those MMPs may stimulate neoplastic progression [7].

### 1.2. Participation of MMPs in progression of melanoma

Melanoma belongs to the group of highly invasive neoplasms with high ability of metastasis through both blood and lymphatic vessels. Some studies suggest that various arrangements of proteolytic enzymes play an important role in progression of melanoma. Those include the plasmin activator system and the extracellular matrix group of metalloproteinases. Melanoma cells have an ability to produce many kinds of ECM metalloproteinases, including MMP-1, -2, -9, -13, -14, as well as other inhibitors: TIMP-1, -2, and -3. It is currently believed that metalloproteinases MMP-2 and MMP-9 play the most important roles in development of melanoma. It was proved that expression and activation of those proteinases is in close relation to the invasive and metastatic melanoma phenotype [50]. Those metalloproteinases are constitutively expressed in the most aggressive forms of melanoma. Their level of expression is strongly related to the atypia of melanocytes and differentiation of cells within the melanocytic nevi [51].

It was found that in case of both human and mouse melanoma cell lines, increased MMP-2, MMP-9 and MMP-14 (MT1-MMP) expression is related to an invasive form of the neoplasm [52]. Increase of MMP-2 is highly related to blood vessel metastasis. Clark's and Breslow's classification shows relation between a high level of MMP-2 expression and low survivability index, which is particularly typical for male patients [50]. Studies carried out by Zucker and Cao [53] proved that MMP-2 is undetectable *in situ* in human melanoma, however high level of an active form of that enzyme is present in the matrix of the primary tumour in advanced stages of melanoma and its metastatic forms. Their results were confirmed by research conducted by Vaisanen et al. [54], who proved that intensified expression of MMP-2 is a typical feature of invasive forms of melanoma and is an unfavourable prognostic factor. Increased

level of MMP-2 expression in mRNA and proteins was also observed in case of the most aggressive melanoma cell lines (MV3, BLM), as well as xenografts derived from them. The pro-MMP-2 activation mechanism has not been fully discovered yet. An active form of MMP-2 directly regulates adhesion of melanoma cells and their proliferation to the ECM, facilitating the processes of migration and invasion [55].

It was also found that increased expression of MMP-14 (MT1-MMP) causes activation of MMP-2 on the surface of melanoma cells. MMP-2 activity is also regulated by presence of TIMP-2. On the one hand, an inhibitor may slow down MMP-2 and in that way limit growth of a tumour and invasion. On the other hand, TIMP-2 may also be directly engaged in activation of MMP-2 through formation of a complex with MMP-14, which is a receptor for MMP-2 on the surface of cells. Therefore, co-ordinated expression of MMP-2, MMP-14 and TIMP-2 leads to activation of MMP-2, while increased concentration of the said metalloproteinases and their inhibitor is also characteristic for other human neoplasms [50, 55].

The hypothesis that there is a dependence between activation of MMP-2 and overexpression of MMP-14 was also proved in the research conducted by Kurschat et al. [56]. They found that in case of abnormal human melanocytes, an increase in expression of both MMP-2 and MMP-14 is related to progression of the neoplasm. An *in situ* zymographic analysis confirmed presence of an active form of MMP-2 in the boundary cells of the tumour and in the surrounding matrix. Moreover, it was proved that the neoplastic cells which produce simultaneously MMP-2 and MMP-14 are often situated on the boundary between the stroma and the front of the invasive tumour cells [56].

Tests conducted on mice deprived of MMP-2 gene confirmed that MMP-2 synthesis is necessary for occurrence of metastasis [50].

Iida et al. [57] found that MMP-14 facilitates invasion of melanoma cells through matrigel, increases their ability to migrate through lamina-1, facilitates growth of the tumour in *in vitro* cell cultures and supports forming of melanoma cell colonies in agarose gel. Moreover, such influence of MMP-14 which causes increase of invasive abilities of melanoma and supports growth of the tumour does not depend on the operation of MMP inhibitors [57].

Participation of MMP-9 in progression of melanoma remains unexplained. So far, contradictory data has only been obtained as to the influence of MMP-9 expression on progression of melanoma. Experiments conducted on melanoma lines derived from advanced stages of the neoplasm proved existence of MMP-9 expression. However, no such occurrence was found in case of cell lines derived from early stages of melanoma [3]. What is more, research carried out by van den Oord et al. [58] shows that, in case of humans, MMP-9 probably undergoes expression only in the phase of horizontal growth of the tumour when it does not exceed 1.6 mm in height. No expression of metalloproteinase-9 was found in metastatic material [58]. Nikkola et al. [59] proved that a high level of MMP-9 in blood serum is typical for patients with vast metastasis and generally shorter survivability time when compared to patients with lower level of MMP-9 in the serum.

In case of experimental models, in which neoplastic tissue was implanted into rodents, MMP-9 was identified within the occurring tumour only when its cells were derived from

advanced melanoma stages [50, 52]. Moreover, in case of organisms that constitutively expressed MMP-9, increased colonization of the lungs and metastasis were observed, while mice without the MMP-9 gene virtually had no secondary neoplastic foci. Those studies suggest that MMP-9, which is produced by both neoplastic and stroma cells, plays an important role in the occurrence of metastasis. Moreover, it is thought that expression of MMP-9 is a feature of neoplastic cells which participate in spontaneous metastasis to the lymph nodes and lungs. Therefore it seems that the process of selection of the cells which create *in vivo* metastasis favours those subclone cells which express MMP-9. As that selection is independent from the microenvironment and is not observed during experimental metastasis, it seems that MMP-9 participates in early stages of metastatic occurrence than in the later states, such as e.g. extravasation [50, 52].

An important role in the development of melanoma is also attributed to MMP-1 and MMP-3. Durako et al. [51] proved that gradation of Type-I and Type-IV collagen, as well as invasion of melanoma cells through matrigel both depend on expression of MMP-1. Nikkola et al. [59] demonstrated correlation between a high level of MMP-1 and MMP-3 in the tissue and a shorter survivability time, as well as a relation between expression of MMP-13 and parenchymal metastases. Increased progression of the neoplastic disease was observed at the beginning of a therapy in case of patients with higher level of metalloproteinases, while in case of patients with melanoma, MMP-13 activity was detected [59].

Due to the properties of TIMPs, it seems that an equilibrium between the level of active MMPs and their inhibitors may be a turning point for neoplastic progression. For example, it was proved that overexpression of TIMP-1, TIMP-2 and TIMP-3 can cause inhibition of melanoma invasion. On the other hand, *in situ* tests show that induction of TIMP-1 and -3 occurs in a late stage of melanoma tumour progression, which is a result of a complex interaction of the MMP/TIMP system. Increased expression of TIMPs can also inhibit metastasis, while simultaneously giving unfavourable prognosis due to TIMPs acting as stimulants to the growth of human melanoma cells [50].

The studies of Wandel et al. [60] show that stroma cells also participate in induction of the proteolytic potential of tumour cells. Therefore, expression of MMPs may occur not only in neoplastic cells but also in the surrounding fibroblasts, which proves participation of those cells in the process of melanoma progression [50]. Wander et al. [60] proved that both fibroblasts located in the direct vicinity of the *in vivo* melanoma lesion, as well as those stimulated with a medium derived from the *in vitro* culture of melanoma cells (the medium contained solvable factors secreted by melanoma cells, such as cytokines and growth factors) showed increased MMP-1 expression of mRNA and proteins. Moreover, a relation between that phenomenon and invasiveness of the tumour was observed.

As the presented data shows, it seems that ECM metalloproteinases play an important role in the development of the neoplastic process, including the melanoma tumour. Anomalies in the expression profile of MMPs not only influence the *in situ* growth of the tumour itself but particularly affect location and the way of induction of the metastatic process. This can suggest that various MMPs may play individual roles in particular stages of melanoma metastasis [52].

## 2. Photodynamic therapy in treatment of melanoma

Photodynamic therapy (PDT) is used in treatment of chosen pre-neoplastic states and early neoplastic stages, as well as for aesthetic and plastic purposes [61]. The basis of PDT is the interaction of laser light (usually long-wave infrared) with photosensitive molecules selectively accumulated in pathologically changed tissue, in presence of oxygen. Such interaction turns those molecules into their cytotoxic forms that, eventually, cause death of the cells, therefore highly selectively destroying pathological lesion in the tissue [62].

A possibility to use photodynamic therapy in treatment of melanoma is currently a subject of research conducted on both cell and animal cultures. Photosensitizers and their derivatives, such as Photofrin, ALA, Porphyrin IX, Vertoporphyrin and others, are currently being assessed or even used in clinical practice in case of other diseases. Simultaneously, very intensive research on newly synthesized compounds is being conducted [63 – 66]. The most attention is drawn to those photosensitizers which have the maximal absorption pattern shifted to the infrared, which allows the light to penetrate the tissue deeper and avoids its absorption by melanin. Laboratory studies also include analyses of mutual influence between molecules of particular photosensitizer and melanin. In case of Chlorin e6 and Polyvinylpyrrolidone complex (FOTOLON) used in our study, research conducted by Parkhots et al. [67] proved that presence of DOPA-melanin (up to 0.1 mg/ml concentration) does not influence the properties of the photosensitizer and those systems do not interact with each other, do not form any bonds nor complexes. However, even quite low concentration of melanin (up to 0.02 mg/ml) in the sample, despite its lack of influence on the rate of singlet oxygen creation in the medium, significantly lowers effectiveness of that reactive form of oxygen. Other studies which assessed mutual interactions of the melanin – photosensitizer - light system described occurrence of so called 'side reactions' [68]. Presence of endogeneous chromophores, including melanin, in surface layers of the illuminated tissue can significantly limit access of the light (especially that of shorter wavelengths). It may also result in so called 'photobleaching', which was confirmed for established parameters of the therapy [68].

First clinical trials with use of PDT in patients with melanoma are currently being conducted. However, the number of reports with successful application of that kind of therapy is still small. Due to very limited knowledge of the molecular and long-term effects of such therapy, the most common but still casuistic reports are related to application of PDT in the palliative therapy of metastatic melanoma. One such case was described by Fasanell and McGrath [69]. Those researchers proved effectiveness of the PDT therapy in treatment of melanoma metastasis to the duodenal ampulla. The therapeutic effect was immediately observed and lasted for a long time (13 months). Unfortunately, further observation was impossible due to death of the patient caused probably by metastasis to the brain and complications in a form of the intracerebral hemorrhage. Other cases of PDT application in the available literature concern patients with melanoma of the sight apparatus, lesions in the nasal area and sensitive parts of the face. There are also reports of successfully concluded therapies of iris melanoma [70], as well as descriptions of 14 patients with metastatic melanoma (13 of them previously treated with other methods) in which cases a successful elimination of all neoplastic cells was achieved after one treatment with PDT-chlorin e6 (in 8 cases) or after a series of therapeutic procedures (in 6 cases) [71].

## 3. Metalloproteinases in photodynamic therapy of melanoma

Photodynamic therapy (PDT) allows for selective elimination of pathological cells with use of light. The light illuminates chosen, mostly pathological, cells of the tissue which gathers photosensitizing substances. The cause of death of those cells are molecular changes that occur during the therapy. However, a question arises: if use of sublethal conditions (that depend on susceptibility of cells, their type, concentration of the photosensitizer, presence of oxygen or dose of radiation) does not increase resistance of the pathological cells to those conditions, leading to occurrence of a malignant phenotype and, in that way, increasing the possibility of metastasis.

More and more studies describe a co-relation between the degree of invasiveness of neoplastic cells and changes in expression of metalloproteinases (MMPs) ECM and their inhibitors (TIMs) located in both neoplastic cells and cells of the stroma - connective tissue of the tumour. Due to that, an attempt to assess influence of photodynamic therapy on changes in expression of chosen meta metalloproteinases MMP-2, MMP-9, MMP-14 and their inhibitors TIMP-1 and TIMP-2 was made in two cultures of melanoma: Colo-829 (melanotic type – a line taken from cells isolated from skin) and SH-4 (melanotic metastatic type – a line taken from cells isolated from pleural effusion; initial occurrence - skin; metastatic occurrence – lungs). The influence of photodynamic therapy on expression of chosen metalloproteinases and their inhibitors was analysed in melanoma cell cultures and regular fibroblast cultures (HFF-1 line – normal fibroblasts isolated from skin), the latter were exposed to factors secreted by melanoma cells after photodynamic therapy.

Chlorin e6 – PVP (Fotolon OAO; producer: Biełmedpreparaty) was used as a photosensitizer with concentration of 0.01 mg/ml of the growth medium. The photosensitizer was added to the growth medium in which cultures of a given cell line were grown. After the growth medium and the photosensitizer were added and an incubation period of 1 hour, cell cultures were illuminated with use of PDT-662 laser (Kriomed) with a wavelength of 662 nm in 5J, 10J and 20 J/cm$^2$ doses.

The analysis of chosen genes was carried out at the transcription stage with use of quantitative determination and QRT-PCR method. Determination of mRNA transcripts of the examined genes in whole-cell RNA extracts was conducted with use of a DNA Engine OPTICON™ fluorescence detector (MJ Research). After each amplification cycle, the number of PCR amplicons was determined based on the intensity of SYBR Green I fluorescent dye which binds to double-stranded DNA, a product of RT-PCR reaction, which has an ability to emit fluorescent light. In order to prepare the reacting QRT-PCR mixture, a stock QuantiTect Sybr Green RT-PCR Reagent Kit (Qiagen) was used. Amplimer identification was based on electrophoretic separation in 8% solution of silver-stained polyacrylamide gel.

Specificity of RT-PCR reaction was confirmed for each analysed sample on the basis of a thermal denaturation profile of the amplification product. The denaturation curve was established for a range of temperature between 50°C and 95°C. Rapid decrease in intensity of fluorescence was observed during denaturation of samples at the melting temperature (Tm) of the amplimer which was contained in that gene. This is due to the release of SYBR Green I dye from the double-stranded product of the PCR reaction.

The results of our study, representing a quantitative feature, were checked for normality by the Shapiro–Wilk test for each trial. In all cases, there was no reason to reject the hypothesis of normality. Thus, to verify the research hypothesis, parametric tests were used and the data is expressed as mean values ± standard deviations. Average results of two samples (trials) were compared using Student's *t*-test when the assumption of variance homogeneity was met, or Student's *t*-test with separate estimation of variance in case of the opposite situation. The homogeneity of variance for two samples (trials) was checked using Snedecor's *F*-test or Levene's test. Comparison of the average of one distinguished group (control) with the remaining experimental averages was made using Dunnett's test, after previously determining (by means of the *F*-test in variance analysis) the statistical significance of differences between analysed averages. When comparing the averages of at least three samples (trials), analysis of variance (ANOVA) was performed. The results for $p < 0.05$ were considered statistically significant. Statistica PL version 8 software was used for the statistical analysis.

The results of the study showed expression of mRNA in *MMP-2*, *MMP-9* (on a very low level), as well as *MMP-14* and *TIMP-2* of SH-4 metastatic melanoma cells. Only MMP9 and TIMP-2 transcripts were detected in Colo-829 melanoma cells (Table 1).

| Melanoma cell line | MMP-2 [mRNA copy number/1 µg of total RNA] | MMP-9 [mRNA copy number/1 µg of total RNA] | MMP-14 [mRNA copy number/1 µg of total RNA] | TIMP-1 [mRNA copy number/1 µg of total RNA] | TIMP-2 [mRNA copy number/1 µg of total RNA] | β-aktyna [mRNA copy number1/µg of total RNA] |
|---|---|---|---|---|---|---|
| Colo-829 | - | 527 | - | - | 47255 | 3922322 |
| SH-4 | 860 | 57 | 558459 | - | 1855586 | 12604750 |

**Table 1.** The mRNA copy number of β-actin, MMP-2, MMP-9, MMP-14 and TIMP-1 and TIMP-2 in melanoma cultures estimated by QRT-PCT method.

However, the results that show limited expression of MMPs in *in vitro* melanoma cell cultures are not surprising. It was even suggested that neoplastic cells themselves do not produce all proteolytic enzymes necessary to initiate the invasion and metastasis [72-74]. Some of those enzymes are probably produced exclusively by stimulated stroma cells [60]. For example, Wandel et al. [60] proved lack of MMP-1 expression in *in vitro* melanoma cell culture lines derived from the primary tumour and the following lines: A365, SK-Mel-13 and MeWo. Whereas Roomi et al. [ADD CITATION] confirmed that some MMP-2 and -9 expression cannot be observed in some *in vitro* types of carcinoma cells. However, Hofmann et al. [55] examined changes in expression of mRNA, MMP-1, -2, -3, -9 metalloproteinases and TIMP-1 and -2 inhibitors in a human model of *in vitro* and *in vivo* melanoma xenograft. The xenograft comprised eight melanoma cell lines with various intensity of maliciousness. Those experiments proved that mRNA level expression of MMP1 occurred in all melanoma cell lines. However, protein-level expression analysed with Northern Blot method could not be confirmed

which may suggest that the amount of MMP-1 in those cells was very small [55]. Presence of mRNA and MMP-2 protein was confirmed in all cell lines, however the highest level of expression, both in latent and active MMP-2 form was identified in the cells with the most malicious phenotype, those which were able to create metastatic foci – MV3 and BLM [55]. Presence of *MMP3* transcripts was confirmed only in three lines: in both most malicious (MV3 and BLM) and in the cell line 530, which did not have metastatic features. However, presence of MMP-3 protein characterized only MV3 and BLM lines. None of studied melanoma cell lines showed MMP-9 expression, yet mRNA and TIMP-1 and -2 protiens were identified in all of them [55]. Therefore, it seems that only the most aggressive melanoma cell lines demonstrate *in vitro* expression of most metalloproteinases, which determine their invasive abilities.

Results of the research for *MMP-2, MMP-9* and *MMP-14* mRNA, as well as *TIMP-1* and *TIMP-2* in SH-4 melanoma cells are shown in Table 1. They can confirm high invasiveness of those cells.

Photodynamic reaction conducted in Colo-829 and SH-4 melanoma cell lines with use of Fotolon causes inhibition of expression on the mRNA level for those metalloproteinases identified above or at least significantly decreases its value. It makes further analysis of influence of therapeutic conditions on expression of chosen *MMPs* and *TIMPs* in examined *in vitro* melanoma cell lines impossible and focuses the researchers' attention on fibroblasts stimulated with a growth media derived from melanoma cell cultures treated with PDT. For it has been confirmed that the invasive and metastatic processes occurring in tumours are results of ECM destruction caused by MMPs, which are produced by both melanoma and stroma cells. Neoplastic cells, due to secretion of various factors, such as growth factors and cytokines, can stimulate neighbouring fibroblasts, which form the stroma tissue of the tumour, to produce and change the level of expression of metalloproteinases [73]. Moreover, results of various studies suggest even that neoplastic cells themselves do not produce all proteolytic enzymes necessary to initiate the invasion and metastasis [72-74]. Some of those enzymes are probably produced only by stimulated stroma cells [60]. Wandel et al. [60] proved that fibroblasts gathered around the melanoma cells have an increased level of *MMP1* expression in comparison to those fibroblasts which have no contact with neoplastic cells. Stimulation of the fibroblasts who did not have any contact with neoplastic cells with the growth medium gathered from melanoma cell cultures leads to over 20 times higher number of *MMP1* transcripts when compared to unstimulated fibroblasts. Those have only three times higher level of expression when compared to rhe SK-Mel-13 line melanoma cells. Increase of *MMP1* expression was also observed on the protein level. Wandel et al. [60] proved statistically significant amounts of proteins between the unstimulated fibroblast cultures and those cultures which were stimulated with the growth medium gathered from melanoma cell cultures. Moreover, Zhang et al. [74] proved that MMP-2 and MMP-14 (MT1-MMP) metalloproteinases produced by fibroblasts attribute to higher invasiveness of *in vitro* HNSCC tumour (Head and Neck Squamous Cell Carcinoma) and stimulate its *in vivo* growth.

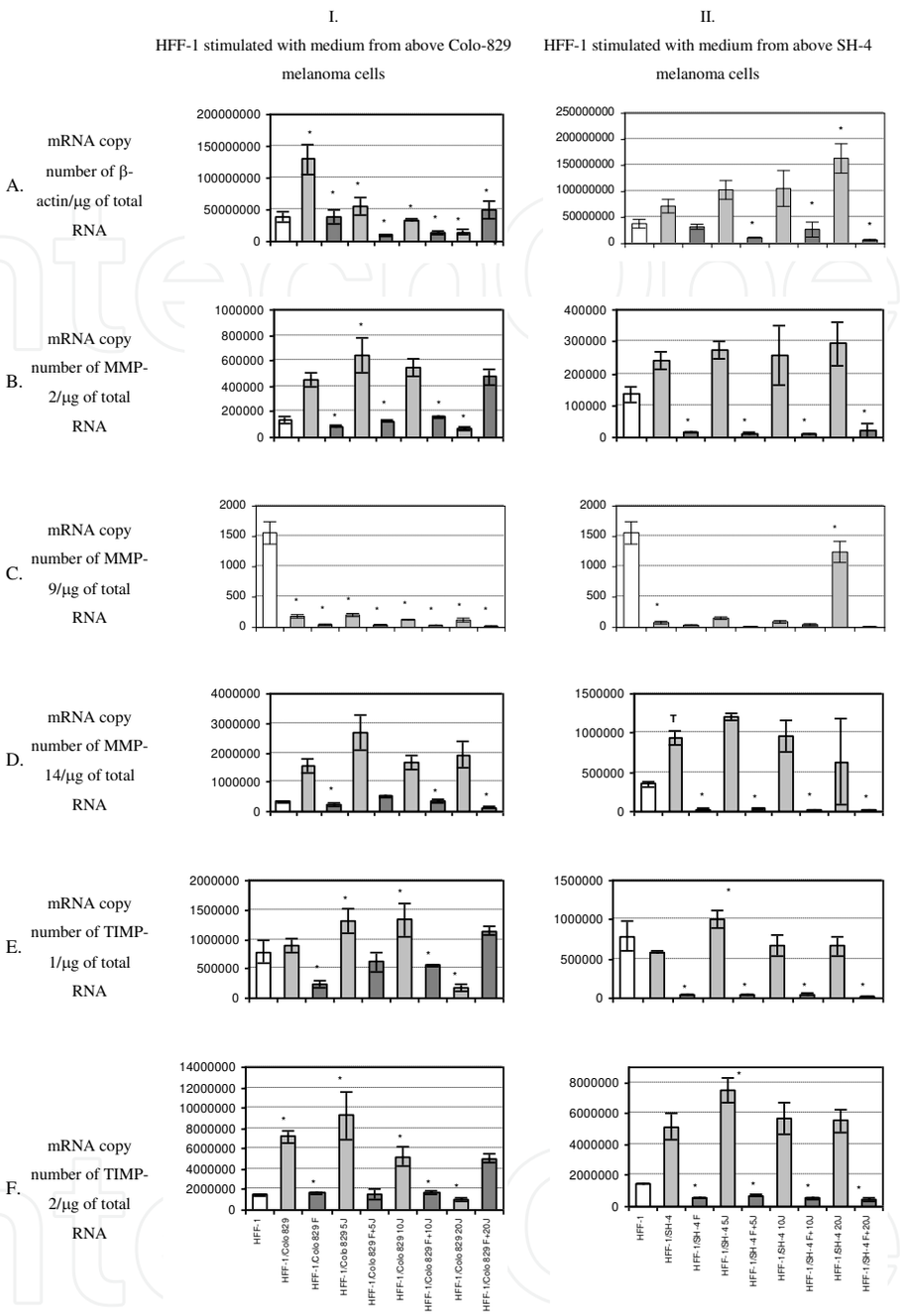


**Figure 1.** Comparison of (A) β-actin, (B) MMP-2, (C) MMP-9, (D) MMP-14, (E) TIMP-1, and (F) TIMP-2 gene expression at transcription level among fibroblast cultures HFF-1 stimulated with medium from above melanoma cultures from Colo-829 and SH-4 cell lines before and after PDT. The first and the second column (column I and II) show the changes of all chosen genes expression in the fibroblast cultures HFF-1 stimulated with medium from above melanoma cultures from appropriately Colo 829 and SH-2 cell lines. The statistically significant results are marked with the symbol „*" ($p<0,05$).

Results obtained for *MMP-2, MMP-9* and *MMP-14* metalloproteinases and their TIMP-1 and TIMP-9 inhibitors confirm processes observed in SK-Mel-13 melanoma line [60], including the

influence of factors secreted by melanoma cells on expression of metalloproteinases (at the mRNA level) and their inhibitors by fibroblast cells in *in vitro* conditions. The conducted research showed general growth of *MMP-2* and *MMP-14* metalloproteinase transcripts, as well as *TIMP-2* inhibitor, in fibroblast cells stimulated by the growth medium gathered from both Colo-829 and SH-4 melanoma cultures. However, statistical significance of the growth of expression on the mRNA level occurs only in the case of *TIMP-2* in fibroblast cultures stimulated by the growth medium gathered from Colo-829 (HFF-1/Colo-829) melanoma cultures ($p < 0.05$). In case of fibroblast cultures stimulated by the growth medium gathered from Colo-829 (HFF-1/Colo-829) and SH-4 (HFF-1/SH-4) cultures, a statistically significant decrease of the number of MMP-9 metalloproteinase transcripts was also observed ($p < 0.05$). In most cases and independently from the applied radiation dose (5 J/cm$^2$, 10 J/cm$^2$ and 20 J/cm$^2$), stimulation of *in vitro* fibroblasts by the growth medium gathered from the melanoma cell cultures which were treated with PDT laser in presence of a photosensitizer causes a decrease of expression on the mRNA level in the examined *MMPs* and *TIMPs* in a statistically significant way ($p < 0.05$) (Fig. 1).

Moreover, in case of normal fibroblast cultures stimulated with the growth medium gathered from Colo-829 melanoma cell cultures that were previously treated with PDT, an increase of the ratio between the number of *MMP2*/*TIMP2*/*MMP14* transcript copies can be observed when compared to HFF-1/Colo-829 cultures (Table 2, Fig.2).

| Stimulated fibroblast cell cultures HFF-1 | The proportion of mRNA copy number: MMP-2 : MMP-14 : TIMP-2 | | | Interaction between the MMP-14:TIMP-2 proportion |
|---|---|---|---|---|
| **HFF-1** (nonstimulated control cell culture) | **1 :** | **3 :** | **11** | **-** |
| **HFF-1/Colo-829** | **1 :** | **3 :** | **16** | **p = 0,0038** |
| HFF-1/Colo-829 F | 1 : | 3 : | 19 | |
| HFF-1/Colo-829 5J/cm$^2$ | 1 : | 4 : | 14 | p = 0,6450 |
| HFF-1/Colo-829 F+5J/cm$^2$ (PDT) | 1 : | 4 : | 13 | |
| HFF-1/Colo-829 10J/cm$^2$ | 1 : | 3 : | 10 | p = 0,1832 |
| HFF-1/Colo-829 F+10J/cm$^2$ (PDT) | 1 : | 2 : | 10 | |
| HFF-1/Colo-829 20J/cm$^2$ | 1 : | 29 : | 15 | **p < 0,0001** |
| HFF-1/Colo-829 F+20J/cm$^2$ (PDT) | 4 : | 1 : | 11 | |
| **HFF-1/SH-4** | **1 :** | **4 :** | **21** | **p = 0,0086** |
| HFF-1/SH-4 F | 1 : | 1 : | 34 | |
| HFF-1/SH-4 5J/cm$^2$ | 1 : | 4 : | 27 | **p < 0,0001** |
| HFF-1/SH-4 F+5J/cm$^2$ (PDT) | 1 : | 3 : | 53 | |
| HFF-1/SH-4 10J/cm$^2$ | 1 : | 4 : | 22 | **p = 0,0060** |
| HFF-1/SH-4 F+10J/cm$^2$ (PDT) | 1 : | 2 : | 42 | |
| HFF-1/SH-4 20J/cm$^2$ | 1 : | 2 : | 19 | p = 0,8270 |
| HFF-1/SH-4 F+20J/cm$^2$ (PDT) | 1 : | 1 : | 19 | |

**Table 2.** The comparition of the proportion of mRNA copy number of MMP-2, MMP-14 and TIMP-2 (MMP-2:MMP-14:TIMP-2) in the fibroblast cell cultures HFF-1 stimulated with media from above melanoma cultures from Colo 829 and SH-4 cell lines before and after PDT. The table shows also the interaction between the MMP-14:TIMP-2 proportion in case of pairs of the cell lines: with and without the Photolon (F) addition (the statistically significant results – interactions - are when p<0,05).

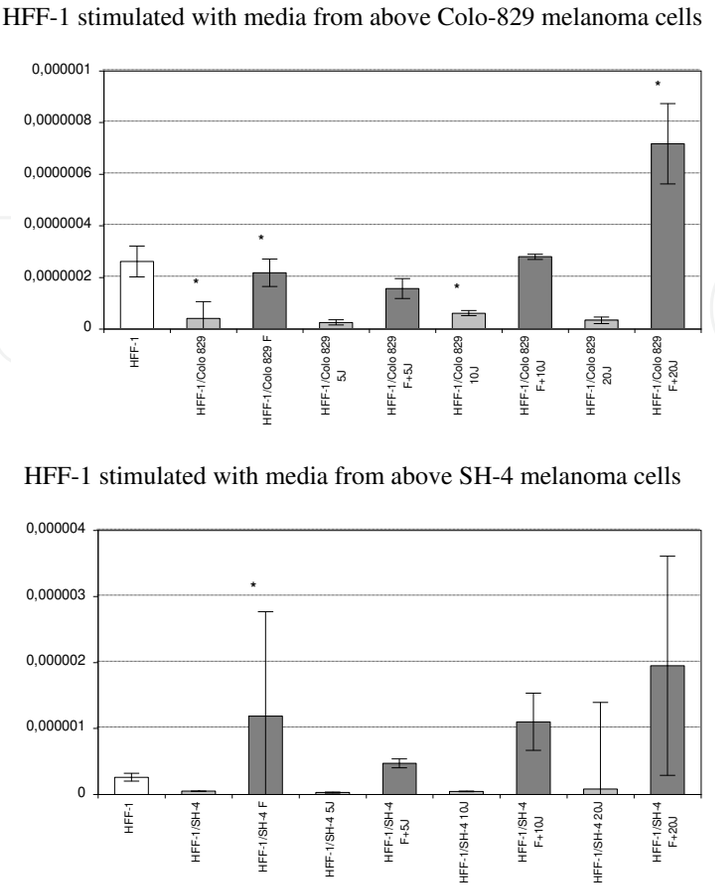


**Figure 2.** Comparison of the MMP-2/MMP-14/TIMP-2 mRNA copy number ratio among fibroblast cultures HFF-1 stimulated with media from above melanoma cultures from Colo 829 and SH-4 cell lines before and after PDT. The statistically significant results are marked with the symbol „*" ($p<0,05$).

Appropriate ratio of *MMP2*/*TIMP2*/*MMP14* gene transcripts guarantees correct course of MMP-2 activation. Increased MMP-14 expression causes activation of MMP-2 on the surface of melanoma cells. Activity of MMP-2 is also regulated by presence of TIMP-2. On the one hand, that inhibitor can influence MMP-2 and, in that way, limit growth of the tumour and invasiveness. On the other hand, TIMP-2 may be engaged in a direct way in activation of MMP-2 by forming a complex with MMP-14, which is a receptor for MMP-2 on the surface of cells. Therefore, only coordinated expression of MMP-2, MMP-14 and TIMP-2 causes activation of MMP-2 [50, 55].

It was also observed during gene tagging that a statistically significant decrease in the number of transcripts of examined genes ($p < 0.05$) in fibroblasts occurred due to stimulation with the growth medium gathered from those melanoma cell cultures which were not treated with PDT but had a photosensitizer added to them. Therefore, Fotolon alone probably causes so significant changes in synthesis and secretion of factors produced by neoplastic cells and later present in the growth medium that it also influences expression of *MMP* genes and fibroblast *TIMPs* (Fig. 1).

The research of expression of MMP-2, MMP-9 and MMP-14, as well as TIMP-1 and TIMP-2 in Colo-829 and SH-4 melanoma cells conducted and described in our study proves that *in vitro* photodynamic therapy of melanoma can cause changes in expression of *MMPs* and *TIMPs*. This may not happen in the melanoma cells themselves but certainly occurs in the connective stroma tissue of the tumour. Moreover, melanoma cells proved to be sensitive to both laser light and the photosensitizer alone.

The analysed ratio of MMP/TIMP copies is also significant, as it may influence later synthesis of proteins and effective activation of metalloproteinases. In most cases, the ratio decreases, which is particularly noticeable in case of cells stimulated with the growth medium from Colo-829. Due to the characteristics of TIMPs, it seems that the equilibrium between the level of active MMPs and their inhibitors may be a turning point in neoplastic progression. As noticed in the literature [50], overexpression of TIMP-1, TIMP-2 and TIMP-3 may be the cause of both inhibition of proteolysis and invasion of melanoma. However, *in situ* research proved that induction of TIMP-1 and -3, which occurs in a late stage of progression of melanocytic tumours, is mainly a result of a complex interaction within the MMP/TIMP system. Therefore, increased expression of TIMPs may inhibit the metastatic process, at the same time giving a bad prognosis [50, 59].

Demonstrated results of the study are related to the influence of PDT therapy on *in vitro* melanoma cultures. Unfortunately, this does not allow for a direct transfer of the observed processes into *in vivo* conditions. Therefore, those results cannot be the only premise to forecast changes of the phenotype in various types of melanoma cells treated with PDT in *in vivo* conditions, as the observed changes in parameters that describe the invasive potential of the tumour do not have to be related only to the speed of its growth and *in vivo* metastasis [74]. In order to understand better the influence that photodynamic therapy has on melanoma cells and the mechanisms triggered by the influence of laser light and a photosensitizer, it is necessary to conduct further, more detailed research. It is certain that changes of expression of *MMPs* and *TIMPs* on the protein level have to be examined in more detail. It is possible that other than mentioned in this study *MMPs* and *TIMPs* may influence the studied processes, depending on a type of melanoma in question. Moreover, the research needs to be conducted in both *in vitro* and *in vivo* conditions. Only after such extended amount of data is collected, a more detailed analysis and comparison of influence of PDT parameters can give a picture of effectiveness and safety of that kind of therapy on melanoma tumour cases.

## 4. Summary

Changes in transcriptional activity of *MMP2, MMP9, MMP14* genes, as well as *TIMP1* and *TIMP2,* in Colo-829 and SH-4 lines of cell cultures suggest that photodynamic therapy does not contribute to an increase of invasiveness of melanoma cells in relation to proteolytic enzymes of the extracellular matrix, even in sublethal conditions.

However, it should be noted that:

- presented results of *MMP2, MMP9, MMP14*, as well as *TIMP1 and TIMP2* expression are related only to the first stage of expression of examined genes (transcription), while the final effect (which is the amount of active form of enzyme proteins coded by those genes) also depends on a number of other factors,
- obtained data is valid for cells cultured in *in vitro* conditions, therefore, in order to formulate an explicit conclusion as to harmlessness of photodynamic therapy in relation to invasiveness of melanoma cells treated with PDT, this study should be treated as a starting point and similar research should be conducted in in vivo conditions.

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