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THE INFLUENCE OF OPIOID PEPTIDES ON MATRIX METALLOPROTEINASE-9 AND UROKINASE PLASMINOGEN ACTIVATOR EXPRESSION IN THREE CANCER CELL LINES

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Matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) regulate proteolysis of the extracellular matrix (ECM) and as a consequence are involved in a number of physiological and pathological states, including cancer. A crucial feature of cancer progression and metastasis is the disruption of the ECM and spreading of proliferating cancer cells. Over-expression of MMPs and uPA is common for most types of cancers and correlates well with the adverse prognosis. Compounds able to modulate the activity of these proteolytic enzymes may become important agents in cancer therapy. In the present study, we examined the effect of the μ-opioid receptor selective peptide, morphiceptin, and its two synthetic analogs on mRNA and protein levels of MMP-9 and uPA in three human cancer cell lines: MCF-7, HT-29, and SH-SY5Y. Our findings indicate that in all three cell lines morphiceptin and its analogs attenuated MMP-9 expression and secretion and that this effect is not mediated by opioid receptors but is under control of the nitric oxide system. On the other hand, tested opioids up-regulated uPA levels through a mechanism that involved opioid-receptors. Different pathways by which opioid peptides exert their action in cancer cells can explain their contradictory influence on the level of cancer markers.

Keywords: **opioid peptides, biochemical markers, ELISA, RT-PCR, nitric oxide synthase.**

Tumor metastasis is a multistep process consisting of several sequential events including detachment of malignant cells from the primary tumor, invasion into the surrounding tissues, intravasation into the circulatory system, adhesion to vascular endothelial cells at distant sites, and extravasation through the endothelial basement membrane to colonize new tissues. The most critical step of metastasis formation is the invasion of cancer cells through the extracellular matrix (ECM), which surrounds the endothelium or creates the basement membrane of epithelial tissue in different organs [1]. The key proteases that are involved in the degradation of ECM are plasmin, uPA and matrix metalloproteinases (MMPs) [2, 3], especially MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which possess the ability to degrade type IV collagen, one of the major components of basement membranes. Expression and activity of all of these enzymes undergo changes in many pathological conditions, including inflammation, degenerative disorders, and cancer. A simple relationship has been established between overproduction of MMPs in tumor cells and cancer progression [4, 5]. Similarly, uPA is believed to mediate cancer dissemination by catalyzing the ECM degradation, thus allowing malignant cells to spread in an uncontrollable way to distant sites. uPA levels have been shown to be up-regulated in most types of cancer [6–9] and correlate well with an adverse prognosis [10–12].

Indeed, preclinical experiments have demonstrated that interference with the proteolytic pathways in cancer cells may inhibit their invasive activity in *in vitro* assays and significantly decrease their invasion and metastatic spreading in animals and humans [13–15]. These observations prompted different studies with various inhibitors designed to block the proteolytic activity of enzymes involved in the ECM degradation and can open a new area for the use of such compounds as therapeutic agents.

Opioids are often administered to cancer patients in order to relieve pain. Presence of opioid receptors on tumor cells has prompted experimental studies investigating the effect of opioids on various regulatory factors that can affect cell proliferation and their migration potential.

This study was designed to test the effect of three opioid peptides, morphiceptin and its two analogs, on MMP-9 and uPA expression and secretion levels in

Abbreviations: ECM – extracellular matrix; eNOS – endothelial nitric oxide synthase; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; MMPs – matrix metalloproteinases; MMP-2 – gelatinase A; MMP-9 – gelatinase B; NO – nitric oxide; PBS – phosphate buffered saline; uPA – urokinase plasminogen activator.

The text was submitted by the author(s) in English.

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three cancer cell lines, MCF-7, HT-29 and SH-SY5Y, and to elucidate the possible mechanism of their action in cancer cells.

EXPERIMENTAL

Opioid peptides. Morphiceptin (Tyr-Pro-Phe-Pro-NH_2) and its two analogs, [Dmt[1], D-Ala[2], D-1-Nal[3]]morphiceptin (analog 1) and [Dmt[1], D-NMeAla[2], D-1-Nal[3]]morphiceptin (analog 2) were synthesized in our laboratory using a standard solid-phase method, as described previously [16].

Cell cultures. The MCF-7 human breast adenocarcinoma, HT-29 colon and SH-SY5Y neuroblastoma cancer cell lines were purchased from the European Collection of Cell Cultures (ECACC). All cell lines were cultured according to the manufacturer's instructions in culture mediums supplemented with gentamycin (5 μg/mL) and 10% heat inactivated fetal bovine serum (both from "Biological Industries", Israel). Cells were maintained at 37°C in a 5% CO_2 atmosphere and grown until they were 80% confluent.

Incubation with opioids. The MCF-7, HT-29 or SH-SY5Y cells (5×10^4 cells/mL) were seeded in 25 ml cell culture flasks in 10 mL of standard growth medium. After 24 h, the growth medium was replaced by a fresh growth medium supplemented with the tested compounds to a concentration of 0.1 μM. Cells incubated without a tested compound were used as a control. After 48 h of incubation, the cells for mRNA isolation were washed twice with phosphate buffered saline (PBS; "Invitrogen", USA) to remove added compounds and were then harvested by trypsinolysis. The cells were frozen in RNAlater ("Sigma-Aldrich", USA) and kept at −80°C till further experiments. For the determination of MMP-9 and uPA protein levels in the medium, the culture supernatant was collected, cleared by centrifugation and stored at −20°C.

Quantitative real-time PCR assay. Total RNA was extracted from the MCF-7, SH-SY5Y or HT-29 cells using a Total RNA Mini Kit ("A&A Biotechnology", Poland) according to the manufacturer's protocol. The concentration and purity of the isolated RNA were determined spectrophotometrically at 260 and 280 nm. cDNA was synthesized using an Enhanced Avian HS RT-PCR Kit and oligo $(dT)_{12-18}$ primers ("Sigma-Aldrich"). Expression levels of the *MMP-9, uPA* and endothelial nitric oxide synthase (*eNOS*) genes, as well as of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), used as a house-keeping gene, were quantified by real-time PCR using an Mx3005P QPCR Systems ("Agilent Technologies, Inc. Santa Clara", USA) according to the manufacturer's instructions for the Brilliant II SYBR Green QPCR Master Mix ("Agilent Technologies, Inc. Santa Clara"). cDNA was amplified with forward and reverse primers that were specific for human *MMP-9*, *uPA*, e*NOS* and *GAPDH* genes. *MMP-9* primer sequences were 5' GACCAATCTCACCGACAGG 3' (forward) and

5' GCCACCCGAGTGTAACCATA 3' (reverse). *uPA* primer sequences were

5' GACCCCCTCGTCTGTTCCCTCCAAG 3' (forward) and 5'CTCTTCCTTGGTGTGACTGCGG 3' (reverse). *eNOS* primer sequences were

5' CAGCCCTCAGAGTACAGCAAGT 3' (forward) and

5' CCATCTCGGGTGTGGTAGGTG 3' (reverse).

As an internal control, *GAPDH* was amplified using primer sequences

5'-GTCGCTGTTGAAGTCAGAGGAG-3' (forward) and

5' CGTGTCAGTGGTGGACCTGAC-3' (reverse).

Real-time PCR reactions were run in triplicate using the following thermal cycling profile: 95°C for 10 min, followed by 40 steps of 95°C for 30 s and 58°C for 1 min and 72°C for 1 min. After 40 cycles the samples were run according to the dissociation protocol (i.e. melting curve analysis). Brilliant II SYBR Green fluorescence emission was registered and mRNA levels were quantified using the critical threshold (Ct) value. Relative standard curves were generated for the tested genes with serial 10-fold dilutions of the cDNA sample. Controls with no cDNA template were included with each assay. The obtained values were normalized relative to the GAPDH transcript levels. All results are presented as mean ±SD.

MMP-9 and uPA secretion. The supernatant collected after treating the cells with the test compound was subsequently analyzed for MMP-9 and uPA protein levels using the MMP-9 ELISA Kit ("RayBiotech", USA) and AssayMax Human Urokinase (uPA) ELISA Kit ("AssayPro, USA), respectively, according to the manufacturer's instructions.

NO secretion. The NO concentration in culture medium after treating the cells with opioids was determined using the Nitric Oxide Quantitation Kit ("Active Motif", USA) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using Prism 4.0 ("GraphPad Software Inc.", USA). Data were expressed as means ± SD. Differences between groups were assessed by a one-way ANOVA followed by a post-hoc multiple comparison Student-Newman-Keuls test. Student's *t*-test was used to compare single treatment means with control means. A probability level of 0.05 or lower was considered statistically significant.

RESULTS

MMP-9 mRNA levels and MMP-9 secretion in cancer cells incubated with opioids

The MMP-9 mRNA expression levels were measured using quantitative RT-PCR. The MCF-7, HT-

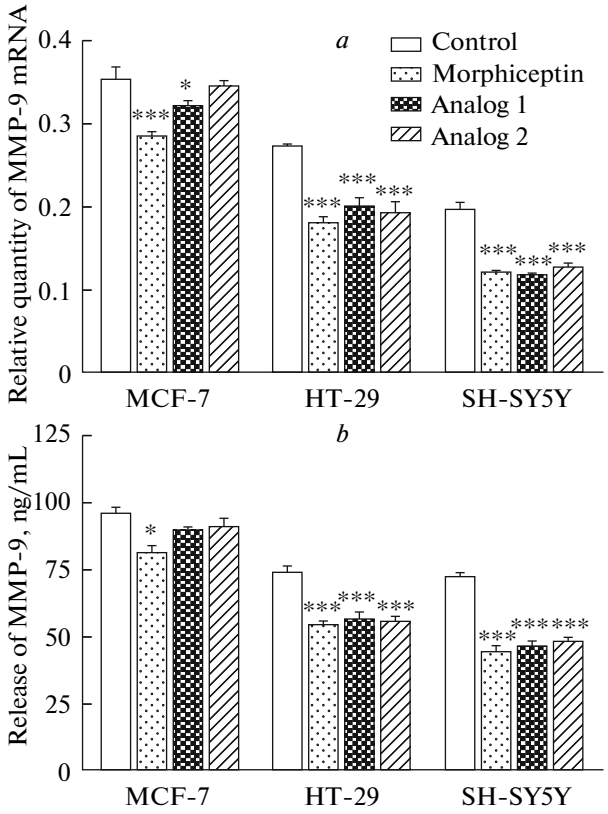

Fig. 1. Effect of morphiceptin and its analogs on MMP-9 expression and secretion. MCF-7, HT-29 and SH-SY5Y cells were incubated for 48 h with morphiceptin or its analogs (0.1 μM): *a* – quantitative real-time PCR analysis of MMP-9 mRNA levels; *b* – the protein levels of MMP-9 (ng/mL) released into the culture medium. Hereinafter, data represented as mean ± SD of three independent experiments and statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student–Newman–Keuls. $*p < 0.05$, $***p < 0.001$ was considered as significantly different from untreated cells, which were regarded as a control.

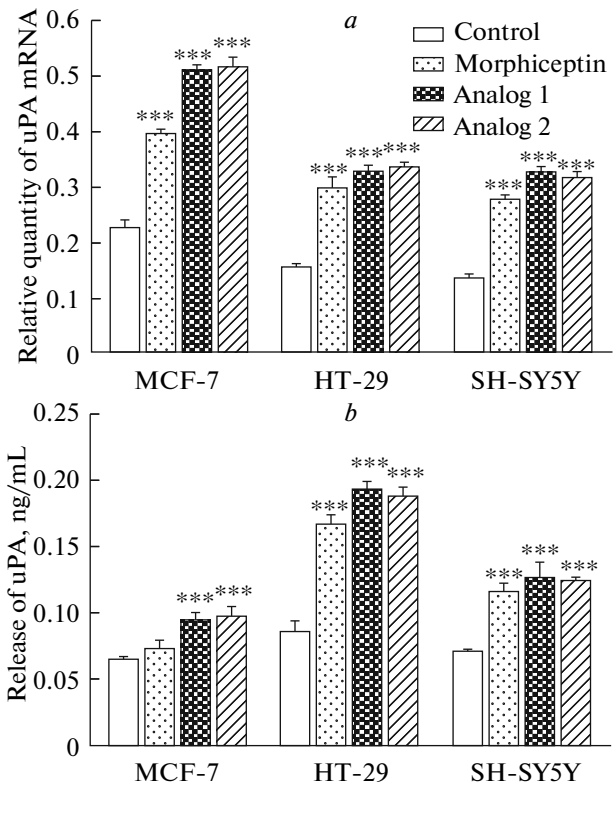

Fig. 2. Effect of morphiceptin and its analogs on uPA expression and secretion. MCF-7, HT-29 and SH-SY5Y cells were incubated for 48 h with morphiceptin or its analogs (0.1 μM): *a* – quantitative real-time PCR analysis of uPA mRNA levels; *b* – the protein levels of uPA (ng/mL) released into the culture medium. $***p < 0.001$ was considered as significantly different from untreated cells, which were regarded as a control.

29 and SH-SY5Y cells were treated for 48 h with morphiceptin or its analogs at a concentration of 0.1 μM. The tested compounds caused a significant down-regulation of MMP-9 mRNA in all tested cell lines, but the effect was the weakest in the MCF-7 cells (Fig. 1*a*). A similar tendency was observed in protein levels: morphiceptin and its analogs decreased the MMP-9 release from the tested cells (Fig. 1*b*).

uPA mRNA expression and uPA secretion in cancer cells incubated with opioids

The uPA mRNA expression was measured using quantitative real-time PCR. The mRNA levels in all tested cell lines treated with morphiceptin and its analogs were significantly up -regulated as compared with the control and the effect exerted by both analogs was stronger than that of morphiceptin (Fig. 2*a*). The uPA protein secretion was also increased in all investigated cancer cell lines (Fig. 2*b*).

Effect of naloxone on morphiceptin induced MMP-9 and uPA mRNA changes in MCF-7 cells

The down-regulation of MMP-9 mRNA levels in MCF-7 cells incubated with morphiceptin was not reversed by an opioid antagonist, naloxone (0.1 μM) that suggests the effect produced by this opioid peptide being not mediated through the opioid receptors. (Fig. 3*a*)

On the other hand, the up-regulation of uPA mRNA expression, induced in MCF-7 cells by incubating with morphiceptin, was completely reversed by naloxone (0.1 μM) (Fig. 3*b*).

Effect of morphiceptin on eNOS mRNA expression and nitric oxide (NO) secretion in MCF-7 cells

As shown above, the down-regulation of MMP-9 mRNA caused by opioids in cancer cells does not involve opioid receptors. Looking for alternative routes, we considered earlier reports indicating that MMPs are under control of NO [17, 18]. The obtained results showed that morphiceptin down-regulated eNOS mRNA expression (Fig. 4*a*) and decreased NO release

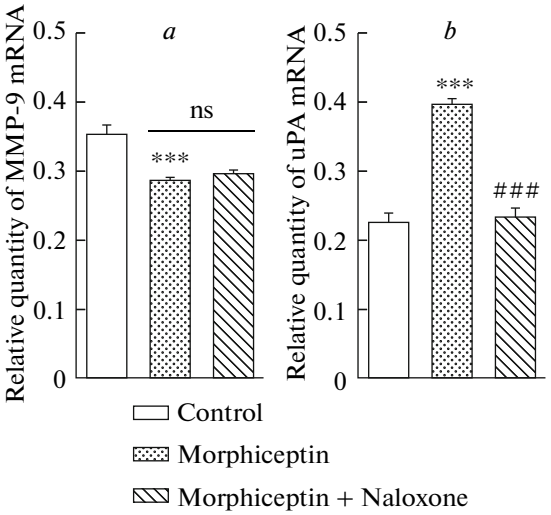

Fig. 3. Effect of naloxone on morphiceptin induced changes in mRNA levels of MMP-9 (*a*) and uPA (*b*) in MCF-7 cells. Cells were co-incubated with morphiceptin (0.1 μM) and naloxone (0.1 μM) for 48 h. Statistical significance was as follows: $^{***}p < 0.001$ *vs* untreated cells regarded as control, $^{\#\,\#\,\#}p < 0.001$ *vs* morphiceptin treated cells.

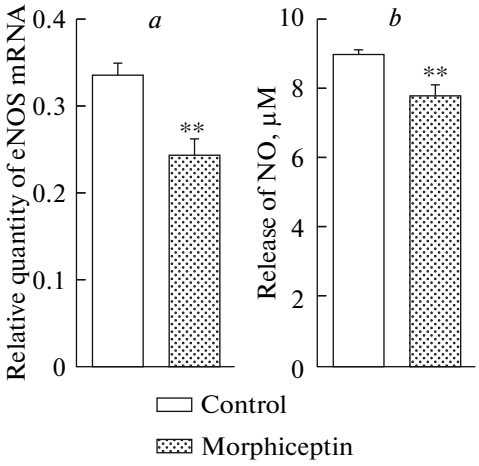

Fig. 4. Effect of morphiceptin on eNOS mRNA expression and NO secretion. MCF-7 cells were incubated for 48 h with morphiceptin (0.1 μM): *a* – quantitative real-time PCR analysis of eNOS mRNA levels; *b* – NO concentration in the culture medium. Statistical significance was assessed using Student's t-test. $^{**}p < 0.01$ was considered as significantly different from untreated cells, which were regarded as a control.

(Fig. 4*b*) from MCF-7 cells, indicating that opioids can influence MMP-9 secretion by modulating NO synthesis.

DISCUSSION

Morphine is often administered to cancer patient to relieve pain. However, there is growing evidence that apart from its analgesic action, morphine can significantly alter tumor growth. In the last decade, numerous studies employing cancer cell lines and experimental animals have been performed to reveal complex mechanisms by which morphine affects tumor cells [19, 20]. Accumulating data suggests that morphine can influence proliferation and migration of tumor cells as well as angiogenesis [21–24].

Recently, it has been demonstrated that the μ-opioid receptor regulates cancer progression in animal models [25–27]. The μ-opioid receptor-knockout mice were shown not to develop significant tumors when injected with Lewis lung cancer cells as did the wild-type controls [21]. Silencing the expression of the μ-opioid receptor in Lewis lung cancer cells inhibited lung metastasis in wild-type mice by about 75%. Finally, infusion of the μ-opioid receptor antagonist, methylnaltrexone, markedly attenuated tumor growth in wild-type mice treated with Lewis lung cancer cells by up to 90% [21]. The experimental data strongly support the hypothesis that the μ-opioid receptor promotes tumor growth and metastasis.

In our earlier studies, the effect of morphine and endomorphin-2, the endogenous ligand of the μ-opiod receptor, on the production of MMP-2 and MMP-9 in MCF-7 cells was examined [28]. As it was demonstrated, both compounds inhibited the expression and secretion of these MMPs, which are known to play an important role in cell invasion by degrading type IV collagen, to a similar degree.

For this study we have chosen other μ-opiod receptor selective ligands, morphiceptin (Tyr-Pro-Phe-Pro-NH_2), a tetrapeptide present in the enzymatic digest of milk protein, β-casein, and its two analogs, [Dmt^1, D-Ala^2, D-1-Nal^3]morphiceptin (analog 1) and [Dmt^1, D-NMeAla^2, D-1-Nal^3]morphiceptin (analog 2), modified by introduction of unnatural amino acids. As it was shown, analog 1 and analog 2 bind to the μ-opioid receptors, respectively, 200- and 400-fold stronger than morphiceptin [16]. Here, we focused on investigations of how these opioids would affect cancer cell invasion in three cancer cell lines, based on their influence on the expression and secretion levels of MMP-9 and uPA.

Results obtained have shown that all three peptides decreased expression and secretion levels of MMP-9 in the tested cell lines. The strongest effect was produced by morphiceptin. On the other hand, tested opioids significantly increased the mRNA and protein levels of uPA and the effects produced by analog 1 and analog 2 were stronger than that exerted by morphiceptin. To elucidate the possible mechanisms of opioid action in cancer cells, the experiments were repeated in the presence of an opioid antagonist, naloxone. The opioid-induced up-regulation of uPA in all three cancer cell lines was antagonized by naloxone that indicates this effect being mediated by opioid receptors. Both analogs, which have a higher affinity to the

μ-opioid receptor than morphiceptin, produced a more pronounced up-regulating effect on uPA. Similar findings were reported by Nylund et al. [29]. Using human colon cancer cell line, HT-29, they demonstrated that morphine affected cell proliferation marginally but caused a marked increase in the secretion of uPA.

On the contrary, the down-regulation of MMP-9 levels by the tested opioid peptides was not reversed by naloxone. However, it was demonstrated before that in a number of systems opioid antagonists did not reverse the effect of opioids on cancer cells. Harimaya et al. [24] showed that naloxone did not influence the inhibitory effect of morphine on the production of MMPs in tumor cells that means opioid receptors to be not involved. Other possible mechanisms were proposed. For example, it may be the interaction of opioids with some intracellular molecules, such as NO, a short-lived free radical gas that can be generated in mammalian cells by a family of NO synthases (NOS). Under physiological conditions cells produce very small amounts of NO. Shariftabrizi et al. [18] reported that in WEHI 164 mouse fibrosarcoma cells MMP-2 was under control of the NO/NOS system and that NOS activity was altered by morphine in a non-opioid receptor-mediated manner.

It is already well recognized that NO up- or down-regulates a heterogenous set of gene products, including protective mediators, chemokines, cytokines, growth factors, hormones and receptors [17]. MMPs are among the preferred targets of NO regulation. Results obtained indicate that opioid peptides inhibited the expression of the eNOS and secretion of NO that suggests the inhibitory effect of the tested opioids on MMP-9 being exerted through the modulation of the NO/eNOS system. The lack of involvement of opioid receptors can also explain why the effect produced by analogs with a much higher affinity for the μ-opioid receptor than morphiceptin was similar or even weaker than the effect produced by morphiceptin itself.

Taken together, obtained data indicate that opioid peptides differentially affect expression and secretion of the ECM degrading enzymes, decreasing MMP-9 levels by a mechanism involving the NO/NOS system and increasing uPA levels through opioid receptors. Differences between cell lines were quite moderate and the same tendency was observed in all three cancer cell types. The different pathways by which opioid peptides exert their action in cancer cells can explain their contradictory influence on the level of cancer markers.

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