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RESEARCH PAPER

Morphine and breast tumor metastasis: the role of matrix-degrading enzymes

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Abstract Opioids including morphine are commonly used in pain management during and after cancer surgery but have been linked to a variety of pro- and anti-tumor effects. In the present study the effect of morphine administration on the localization and growth of breast tumor cells in lungs and the level of extracellular matrix (ECM) proteases were investigated. In a mouse syngeneic model of intravenously inoculated breast cancer cells, morphine administration led to a reduction in the localization and growth of tumors in the lungs and a reduction in circulating matrix metalloproteinase-9 (MMP-9) and urokinase-like plasminogen activator (uPA). To model the involvement of non-malignant cells of the tumor microenvironment in the changes we observed in the level of proteases, we co-cultured breast cancer cells with macrophages, endothelial cells and fibroblasts. We found a significant elevation of matrix proteases as well as matrix protease inhibitors in co-cultures of breast cancer cells with macrophages or endothelial cells. Interestingly, morphine treatment of these co-cultures reduced the level of MMP-9 and increased its endogenous inhibitor, TIMP-1, thereby altering the proteolytic profile. Morphine affected the level of enzymes in co-cultures but not in cells grown individually. This suggests that anti-tumor effects of morphine observed in our in vivo model could be mediated at least in part through modulation of paracrine communication between cancer cells and non-malignant cells in the tumor microenvironment.

Keywords Breast cancer · Endothelial cells · Macrophages · Matrix metalloproteinase-9 · Morphine · Urokinase plasminogen activator

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Introduction

Cancer recurrence after surgical resection of primary tumors is a major challenge in cancer treatment. There is now abundant evidence that perioperative medications could have a significant impact on the outcome of cancer surgery (reviewed in [1]). Morphine is one of the most effective perioperative analgesic drugs and is commonly used in cancer surgery. Evidence suggests that morphine can influence tumor growth, metastasis and angiogenesis in animals [2]. At cellular level it is shown to affect proliferation, survival, migration, adhesion and invasion of multiple cancer cell types [3].

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Tumor growth and metastasis requires reciprocal interactions between tumor cells and stromal cells including immune cells, fibroblasts and endothelial cells. These interactions are mediated through the release of multiple growth factors, cytokines and proteases that enable tumor cell transendothelial and tissue migration and provide a permissive milieu for malignant cells in the secondary organs [4].

We employed a mouse syngeneic model of breast cancer where murine breast tumor cells were intravenously inoculated in immunocompetent mice and subjected them to morphine. Short-term administration of morphine, similar to a perioperative analgesic regimen, decreased the number of tumor foci in lungs and the level of circulating proteases. Due to the significance of tumor-host interaction in the regulation of ECM proteases, we further examined the level of proteases in co-cultures of breast cancer cells with the most prominent non-malignant cells in the microenvironment namely macrophages, endothelial cells and fibroblast. We tested the effect of morphine on the interaction of macrophages or endothelial cells with breast cancer cells.

We hypothesized that morphine administration might affect the colonization of circulating tumor cells in the lungs as primary site of distant metastasis in our model. We further hypothesized that the effect of morphine would be mediated by modulation of the proteolytic profile resulting from interaction of tumor cells with macrophages and endothelial cells.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, RPMI-1640, foetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen (Life Technologies, Mulgrave, VIC, Australia). The following reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, and Australia): bovine skin gelatine type B, bovine milk casein and bovine plasma plasminogen. DBL® morphine sulfate injection was from Hospira (Mulgrave, VIC, Australia).

Cell culture

4T1 murine breast cancer cells were maintained in RPMI-1640 medium supplemented with 5 % (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 % (v/v) sodium pyruvate. RAW264.7 murine macrophages and NIH3T3 murine fibroblasts were maintained in DMEM with 10 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. H5V murine endothelial cells were maintained in DMEM-F12 medium supplemented with 5 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. All cells were kept in a humidified atmosphere containing 5 % CO_2.

Conditioned media preparation

To prepare conditioned media, 80 % confluent cells were rinsed with serum-free medium and placed in serum-free medium for 48 h. The conditioned media were then collected and centrifuged at $1{,}100\times g$ for 5 min. Supernatants were collected and kept at −20 °C until analysed.

Animal studies

All animal experiments have been conducted in accordance with the ethical standards and according to national and international guidelines and were approved by institutional animal ethics committees. 6–8 week old female mice were used. Mice were maintained under 12:12 light/dark cycles, controlled temperature and humidity. Animals had free access to standard food and tap water. To test the effect of morphine on tumor cell retention and growth in lungs, 4T1 cells (2×10^5) in phosphate buffer saline (PBS) or PBS as control were intravenously injected into the tail vein of BALB/c mice. Animals were treated for three days with 10 mg/kg morphine or with saline I.P. every 12 h. Mice were euthanized 18 days after tumor inoculation and their blood was collected, serum samples were prepared and analysed by in-gel zymography. To enumerate metastatic foci in the lungs, the lungs were stained by intra-tracheal injection of 15 % (w/v) India ink solution in water. Lungs were collected, washed and fixed in Feket's solution (62 % ethanol v/v, 3.5 % formaldehyde v/v and 1.5 % acetic acid v/v). White tumor nodules against the black stained lung tissue were counted.

Our studies employ 10 mg/kg morphine twice daily for three days after tumor inoculation. Doses in mg/kg cannot be compared between humans and mice because mice metabolize morphine mainly to μ opioid receptor-inactive morphine-3-glucuronide (M3G), whereas humans convert morphine into both M3G and active morphine-6-glucuronide (M6G), and thus require lower doses for equivalent analgesia. Studies of pain in mice commonly use 10 to 40 and even up to 100 mg/kg intraperitoneal or subcutaneous morphine [5–7]. Importantly, the dose of morphine that we employ in this study leads to serum concentrations in mice similar to the range we use in our in vitro experiments [8, 9].

Co-cultures

4T1 cells and RAW264.7 murine macrophages were cultured alone or together in equal numbers (8×10^4) in

24-well plates. 4T1 cells and H5V endothelial cells or NIH3T3 fibroblasts were cultured alone or together in equal numbers (5×10^4) in 24-well plates. Cells were seeded and maintained in a medium consisting of a 1:1 mix of the medium of each cell line for 24 h. Cells were washed twice and incubated in a 1:1 mix of the same media without serum, added with morphine (0–10 μM). These concentrations of morphine do not affect the viability of any of the cell types used in our in vitro experiments. The 48 h conditioned media were collected, tested for protein content and analysed by gelatine, reverse gelatine and casein–plasminogen zymography. In another set of experiments, 4T1 cells were incubated with the conditioned medium of RAW26.7 macrophages or H5V endothelial cells treated with morphine (0–10 μM). RAW 264.7 cells and H5V cells were also incubated with the 48 h conditioned medium of 4T1 cells. The conditioned media of these co-cultures were analysed for proteases by zymography.

Gelatine, reverse gelatine and casein–plasminogen zymography

Conditioned media and sera were analysed for MMPs and uPA content using gelatine and casein–plasminogen zymography, respectively. An 11 % polyacrylamide gel containing 1 % (w/v) gelatine was used to measure the level of gelatinases (MMP-2 and MMP-9). To measure uPA, 1.5 % (w/v) casein and 0.02 U/ml plasminogen were added to the gel. In order to measure TIMPs, NIH3T3 conditioned medium was added to a 12 % polyacrylamide gel as a source of gelatinase along with 1 % (w/v) gelatine.

Equal amounts of protein were separated by SDS-PAGE. The gels were incubated in a renaturing solution (50 mM Tris, 5 mM $CaCl_2$, and 2.5 % (v/v) Triton X-100) overnight. The gels were rinsed and incubated at 35 °C in a solution containing 50 mM Tris–HCl and 5 mM $CaCl_2$ for 3 h. Gels were stained with staining solution (0.25 % (w/v) Coomassie Blue R-250, 45 % (v/v) methanol, 10 % (v/v) glacial acetic acid) and destained with a solution of 25 % (v/v) methanol, 10 % (v/v) acetic acid until the proteolytic bands appeared clear on a dark background, or the proteolysis inhibition appeared as dark areas against a pale background. The gels were scanned using a high-resolution flatbed scanner and the intensity of the bands was measured with NIH Image J software. Results are expressed as arbitrary units, except in coculture studies where results were expressed as percent of the group representing the most appropriate control.

Statistical analysis

Results are presented as mean values (± SEM). Statistical analysis was performed using Graphpad Prism. Groups were compared using Student's *t* test. One-way or two-way analysis of variance was used as appropriate when more than two groups were compared. $p < 0.05$ was considered as statistically significant.

Results

The presence of tumors increases circulating matrix proteases

We determined the amount of circulating gelatinases and uPA in mice 18 days after tail vein inoculation of 4T1 cells. A significant elevation of the activity of MMP-9 (Fig. 1a, b), and uPA (Fig. 1a, c) was observed in the serum of tumor-bearing mice compared to the control group. The level of MMP-2 was unchanged in tumor compared to no-tumor group (data not shown).

Morphine inhibits the colonization of breast cancer cells in lungs and decreases the level of circulating proteases in mice

BALB/c mice intravenously inoculated with 4T1 breast cancer cells were treated with 10 mg/kg morphine or saline I.P. every 12 h for 3 days. Tumor nodules in the lungs were enumerated 18 days after inoculation (Fig. 2a). The number of metastatic foci in lungs was significantly lower in morphine-treated mice compared to saline-treated mice (Fig. 2b). Serum samples were analysed by zymography (Fig. 3). In tumor-bearing mice circulating levels of MMP-9, but not MMP-2, were significantly reduced by morphine treatment (Fig. 3a, c). Similarly in tumor-bearing mice, the level of uPA was lower in the serum of morphine-treated mice compared to saline-treated mice, while tPA levels seemed unchanged (Fig. 3b, d). In tumor-free mice morphine did not have any significant effect on circulating matrix proteases at day 18.

Interaction of tumor cells with stromal cells remodels their proteolytic profile

To refine our understanding of the interaction between breast cancer cells and stromal cells, we established co-culture models and tested gelatinases, uPA and TIMPs in the conditioned media. 4T1 breast cancer cells were cultured with RAW264.7 macrophages, H5V endothelial cells or NIH3T3 fibroblasts. The 48 h conditioned medium of individual or co-cultured cells was tested using zymography. The level of MMPs, TIMPs or uPA were not different in the co-cultures of 4T1 breast cancer cells with NIH3T3 fibroblasts compared to the level of proteases produced by 4T1 or NIH3T3 cells alone (not shown). However, the

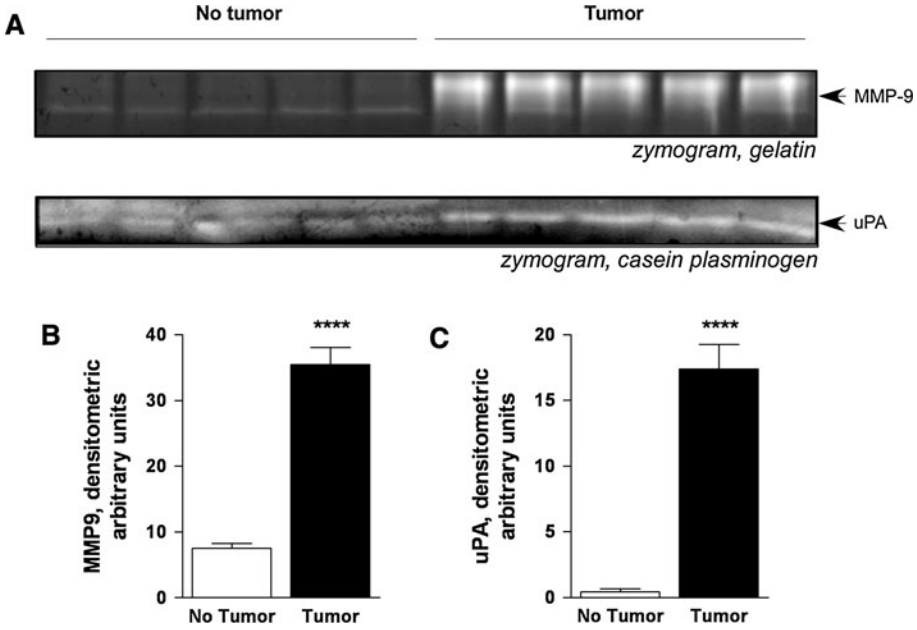

Fig. 1 Tumor-bearing mice have increased circulating matrix proteases. Mice were injected with 4T1 cells in the tail vein and serum collected at euthanasia. MMP-9 was assessed by gelatine zymography, uPA by casein–plasminogen zymography. Representative gels are shown (**a**). Gels were scanned and analysed by densitometry (**b**, **c**). Mean ± SEM is shown, n = 4–8 per group. ***$p < 0.001$, ****$p < 0.0001$

level of MMP-9 and TIMP-1 was significantly higher in the conditioned media of co-cultures of 4T1 cells with macrophages compared to 4T1 cells alone (Fig. 4). MMP-2 and TIMP-2 levels in these co-cultures were not different from that produced by 4T1 cells alone. In co-cultures of 4T1 cells with endothelial cells, MMP-9, MMP-2, TIMP-1, TIMP-2 and uPA were all elevated compared to conditioned media from individual cells, although the increase did not reach statistical significance for MMP-9 (Fig. 4). These results show that interaction of breast tumor cells with stromal cells such as macrophages or endothelial cells alters their proteolytic profile.

Morphine modulates the proteolytic enzyme remodelling induced by co-culture of breast tumor cells with either macrophages or endothelial cells

To assess the effect of morphine on the interaction between breast cancer cells and either macrophages or endothelial cells, co-cultures of breast cancer cells with macrophages or endothelial cells as well as individual cell cultures were exposed to 0.1–10 μM morphine in serum-free medium for a duration of 48 h. The conditioned media were analysed by zymography. Morphine did not alter the level of proteolytic enzymes produced by 4T1, RAW264.7 or H5V cells cultured alone (data not shown). Interestingly, in co-cultures of 4T1 cells with H5V endothelial cells, morphine caused a significant decrease in MMP-9 level as well as a significant increase in the level of TIMP-1, resulting in a considerable decrease in MMP-9/TIMP-1 ratio (Fig. 5). Similar results were observed in co-cultures of 4T1 cells with RAW264.7 macrophages (Fig. 5). These results indicate that morphine can modulate the induction of matrix proteases and their inhibitors caused by the interaction of endothelial cells and macrophages with breast cancer cells. MMP-2, TIMP-2 and uPA levels were not significantly altered by morphine either in cells cultured alone or in co-cultures.

To determine the role of soluble factors released from each cell type on the proteases released by the other, we incubated breast cancer cells with the 48 h conditioned medium of macrophages or endothelial cells—and incubated endothelial cells or macrophages with the 48 h conditioned medium of breast cancer cells to analyze their media by zymography. The most noticeable change was seen with the conditioned media from macrophages, which was capable of inducing a similar increase in MMP-9 production by 4T1 cells as observed when cells were in direct contact (Fig. 6a). To further test whether this paracrine interaction was modulated by morphine, 4T1 cells were placed in the conditioned medium of morphine-treated macrophages. A decrease in MMP-9 and an increase in TIMP-1, resulting in decreased MMP-9/TIMP-1 ratio were detected (Fig. 6b–d). The change was not due to a lower amount of MMP-9 in the conditioned medium of the morphine treated macrophages, or to the direct effect of residual morphine in the conditioned medium from macrophages on the 4T1 cells (Fig. 6e). These results suggest that morphine decreases the ability of macrophages to elicit, via paracrine factor(s), breast cancer cell proteolytic activity.

Discussion

The question of whether morphine might influence tumor growth and metastasis has been hotly debated in recent

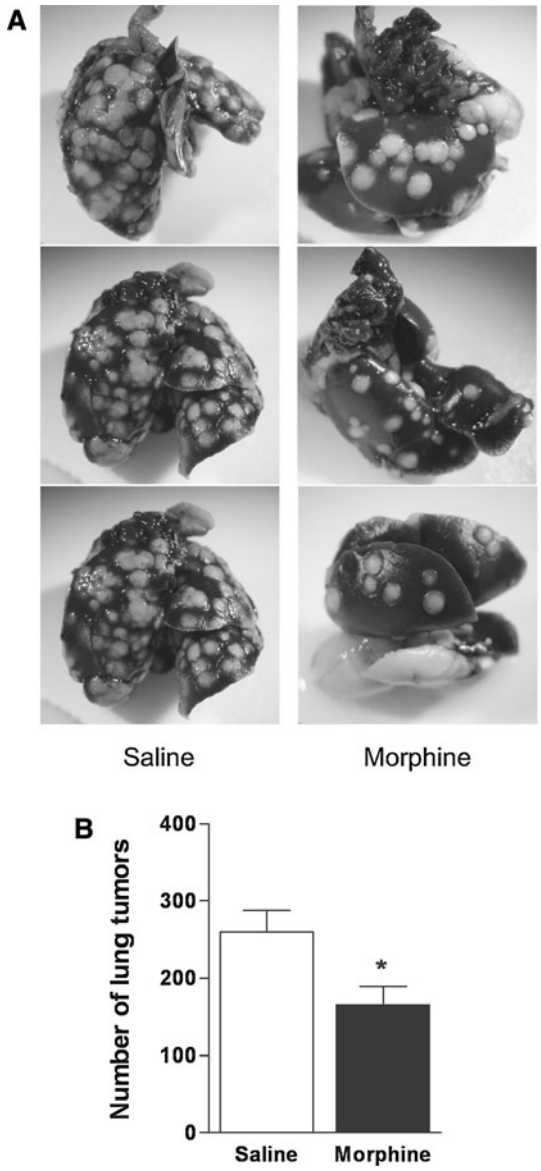

Fig. 2 Morphine reduces breast cancer cell take in the lungs. BALB/c mice were injected with 200 000 4T1 cells via the tail vein. The mice then received saline or morphine I.P. every 12 h for 3 days. The lungs were collected after 18 days, and the number of tumors assessed. **a** Representative pictures of lungs of three mice from each group. **b** Number of lung tumors in each group of mice. Mean ± SEM is shown, $n = 8$ per group, $*p < 0.05$

years. It is of particular importance due to the fact that opioids are widely used in cancer surgery patients. In the present study, we dissected out the influence of morphine on the growth and metastatic potential of breast tumor cells using a combination of in vitro and in vivo assays. Other studies have tested the role of morphine on breast tumor cell seeding in the context of surgical stress response [10], or the effect of long term, sub-analgesic doses of morphine on spontaneous metastasis from tumors [11]. We chose an in vivo model that employed a three day opioid regimen similar to what cancer surgery patients receive, and the direct injection of breast tumor cells in the tail vein to mimic the presence of cancer cells in the circulation during and after tumor ablation.

Administration of morphine to mice decreased retention and growth of breast tumor cells in lungs (Fig. 2). This may be the result of complex, combined actions that morphine has at cellular, organ and systemic levels. Morphine is known to have both direct and indirect effects on tumor cells, influencing their growth, survival, invasiveness and ability to metastasize [2]. In bioassays, morphine administered to mice further has pro-migratory effects that can be demonstrated ex vivo [12]. The effect of morphine on the sympathetic nervous system, immune system, vasculature as well as tumor microenvironment further influences tumor growth and metastasis in the body [13–16]. Our results are in agreement with studies from other groups showing that morphine reduces tumor growth in vivo [15, 17, 18] but in contradiction with studies showing that morphine increases breast tumor growth and metastasis in murine breast cancer models [11, 13]. Explanations for these discrepancies include differences in genetic variations of opioid sensitivity between mouse strains, different experimental conditions, wide ranges of morphine doses, integrity of the mouse immune system, and the presence or absence of pain in the model studied [2, 19]. Our model employs a syngeneic system, and therefore allows immunocompetent mice, which is an advantage considering that morphine affects the immune system via numerous mechanisms that are believed to result in overall immunosuppression. Furthermore, some of the studies showing pro-tumor effects of morphine on breast cancer employed morphine below clinically relevant doses [11, 13, 20] and for longer duration than did our study [11, 13, 14].

Our experiments show that morphine induced lower lung retention and growth of tumor cells introduced into the blood stream of mice via tail vein injection. Experiments in rats have concluded that morphine reduced the metastatic spread of tail vein-injected tumor cells when the animals were subjected to pain and surgical stress [10, 21]. The possibility that morphine acts by alleviating, in our mouse model, pain originating from the inoculation or early metastatic foci cannot be ruled out. Furthermore, opioids cause bronchoconstriction and reduced pulmonary blood flow by sympathomimetic actions and histamine release [22], suggesting that fewer tumor cells might be entrapped in the lungs of morphine-treated mice. However this possibility can be ruled out by the fact that morphine treatment was started more than 12 h after tumor inoculation in our studies.

Our search for a mechanism of action for morphine in our in vivo model led to the discovery that ECM-degrading enzymes such as MMP-9 and uPA, whose production is up-

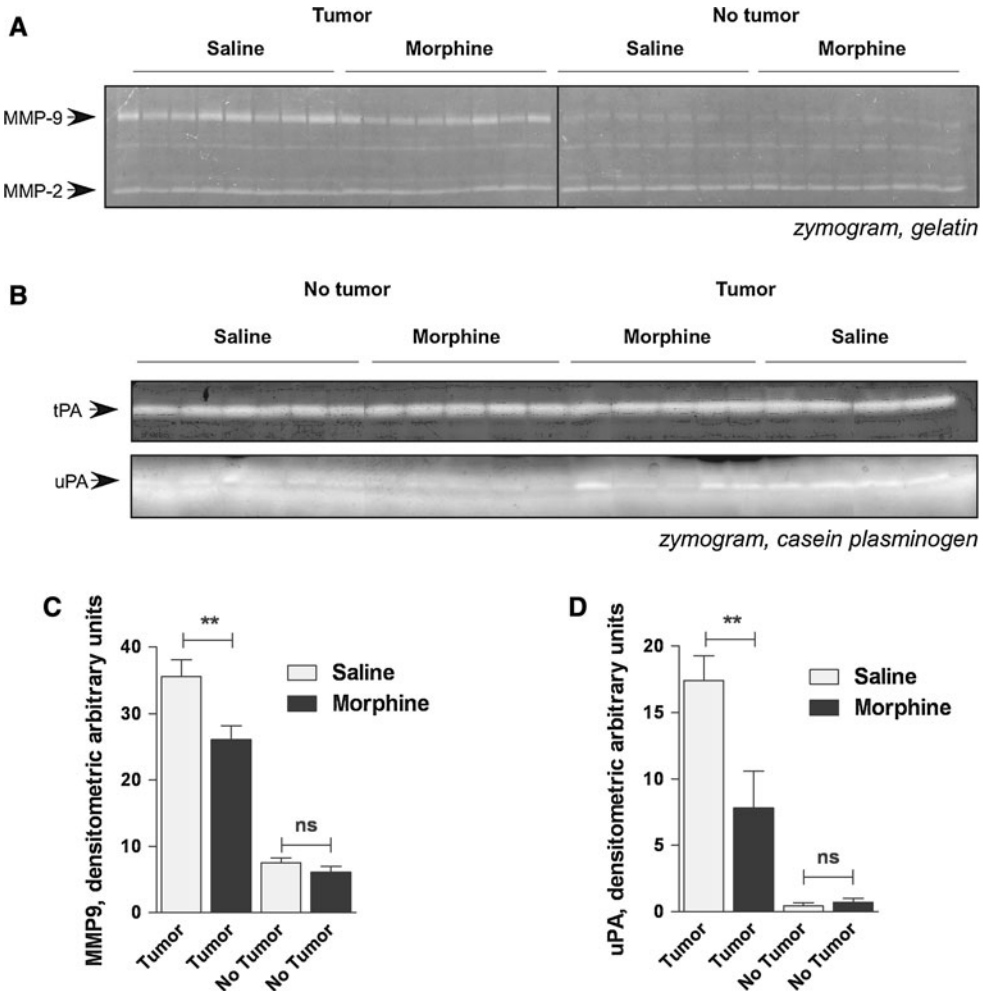

Fig. 3 Morphine administration reduces serum MMP-9 and uPA in tumor-bearing mice in vivo. **a** MMP-9 activity was seen predominantly in the tumor-bearing mice and was decreased by morphine treatment. Activity of a 72 kDa gelatinase, corresponding to MMP-2, was seen in all samples and seemed unaltered by the presence of tumors or the morphine treatment. **b** uPA activity was observed predominantly in the tumor-bearing mice and was decreased by morphine treatment. tPA was seen in all samples and appeared unaltered by the presence of tumors or the morphine treatment. **c** MMP-9 in-gel activity was quantified by densitometry $^{**}p < 0.01$ tumor saline vs tumor morphine. Mean ± SEM is shown, n = 8 per group (two-way ANOVA analysis with Bonferoni multiple comparisons). **d** uPA in gel activity was quantified by densitometry $^{**}p < 0.01$ tumor saline vs tumor morphine. Mean ± SEM is shown, n = 4–6 per group (two-way ANOVA analysis with Bonferoni multiple comparisons)

regulated by tumors, can be modulated by morphine. These proteases regulate tissue invasion and metastasis as well as the recruitment of immunocytes and endothelial cells to the tumor. Furthermore, MMPs regulate cell adhesion, proliferation, differentiation and apoptosis and directly affect tumor growth, angiogenesis and metastasis. uPA on the other hand converts plasminogen to plasmin which activates several enzymes and growth factors important to the microenvironment including MMPs. Activation of uPA receptor (uPAR) on the cell surface also directly affects cell adhesion, migration and proliferation. We found significantly elevated circulating MMP-9 and uPA levels in tumor-bearing mice, in agreement with previous studies [23–25]. However, circulating MMP-2 was unaltered by the presence of tumors. Moreover, our experiments show for the first time that morphine treatment reduces the level of MMP-9 and uPA (but not MMP-2) in tumor-bearing mice.

Expression and activity of ECM proteases is closely regulated by non-malignant cells in the tumor microenvironment including fibroblasts, endothelial cells and macrophages [26]. To characterize the effect of morphine on protease production by breast cancer cells and stromal cells, we employed co-culture systems. These experiments showed that co-culture of breast cancer cells with macrophages or endothelial cells led to the up-regulation of proteases and their inhibitors, resulting in a cell type-specific profile: interaction with macrophages led to increased MMP-9 and TIMPs, while interaction with endothelial cells led to increased MMP-9 (although statistically not

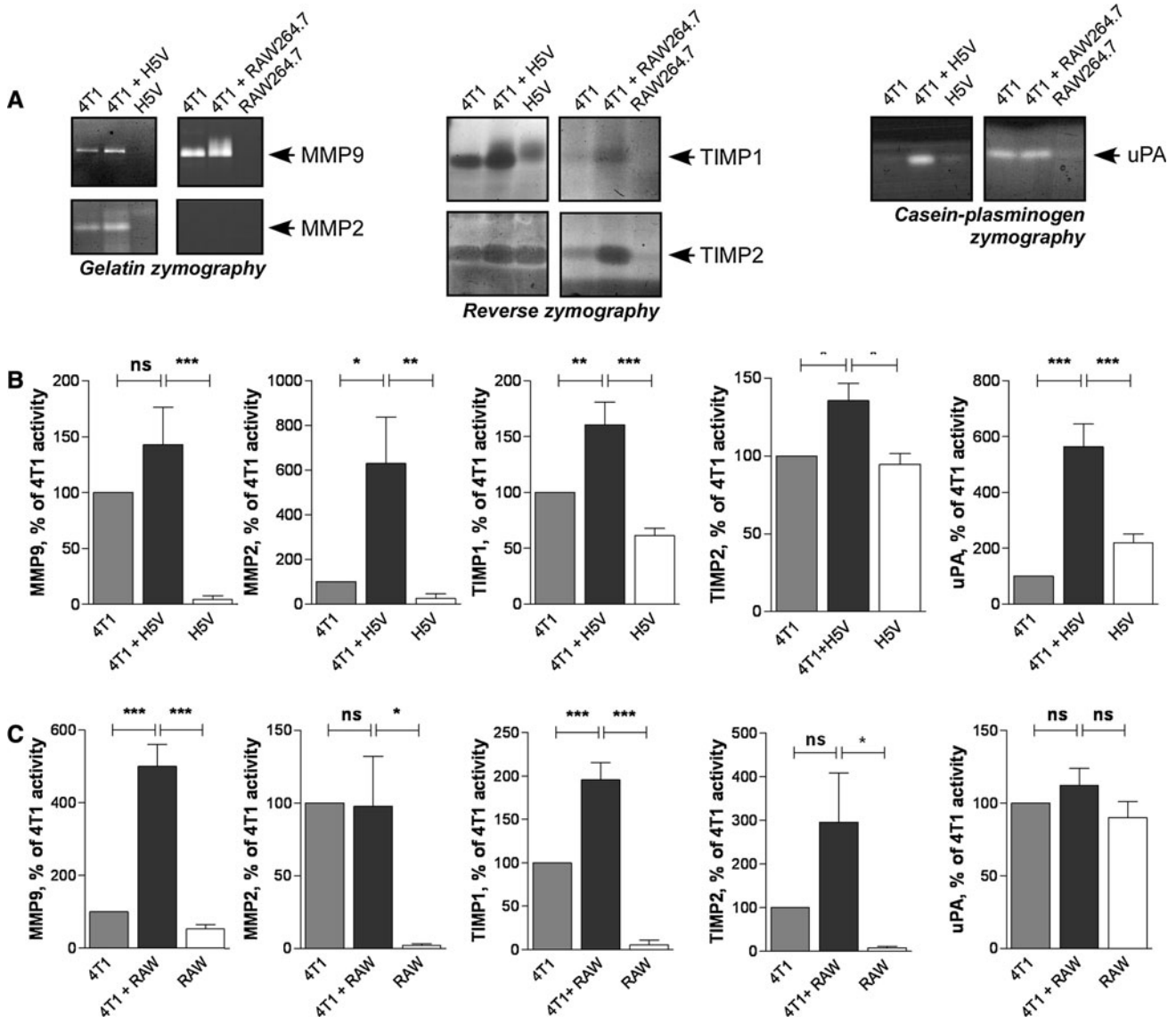

Fig. 4 Interaction between breast cancer and macrophages or endothelial cells in culture modulates the matrix proteolytic profile. Cells were cultured separately or together as indicated and their conditioned medium collected. **a** MMP-9 and 2 were detected by gelatine zymography. TIMPs were assessed by reverse gelatine zymography, while uPA was measured by casein–plasminogen zymography. **b** Quantification of MMPs, uPA and TIMPs in conditioned media of 4T1 cells and endothelial cells grown alone or together. **c** Quantification of MMPs, uPA and TIMPs in conditioned media of 4T1 cells and macrophages grown alone or together. Mean ± SEM is shown, n = 3–10 per group $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

significant) and increased MMP-2, TIMPs and uPA. These changes are in agreement with previous data on the overexpression of MMP-2 and -9 in co-cultures of cancer cells with monocytes and macrophages [27, 28] and MMP-2, MMP-9, TIMP-1 and uPA in the cocultures of cancer cells with endothelial cells [29–31] and could reflect a pro-migratory environment for tumor cells, in line with the published literature on tumor-promoting effects of stromal cells [27, 32–34]. We designed the co-cultures using cells of the same species (namely murine cells) to promote their interaction, but as a consequence we could not differentiate the enzymes produced by each cell type based on their electrophoretic size.

To determine the mechanism of morphine's action in our coculture model, we tested whether contact or cross talk between macrophages and breast cancer cells was required, or if morphine prevented a simple, unidirectional paracrine effect of the macrophages on 4T1 proteolytic potential. Our results show that morphine prevents the production or the action of factor(s) produced by macrophages that induce increased proteolytic ability of breast cancer cells. This effect of morphine was less significant

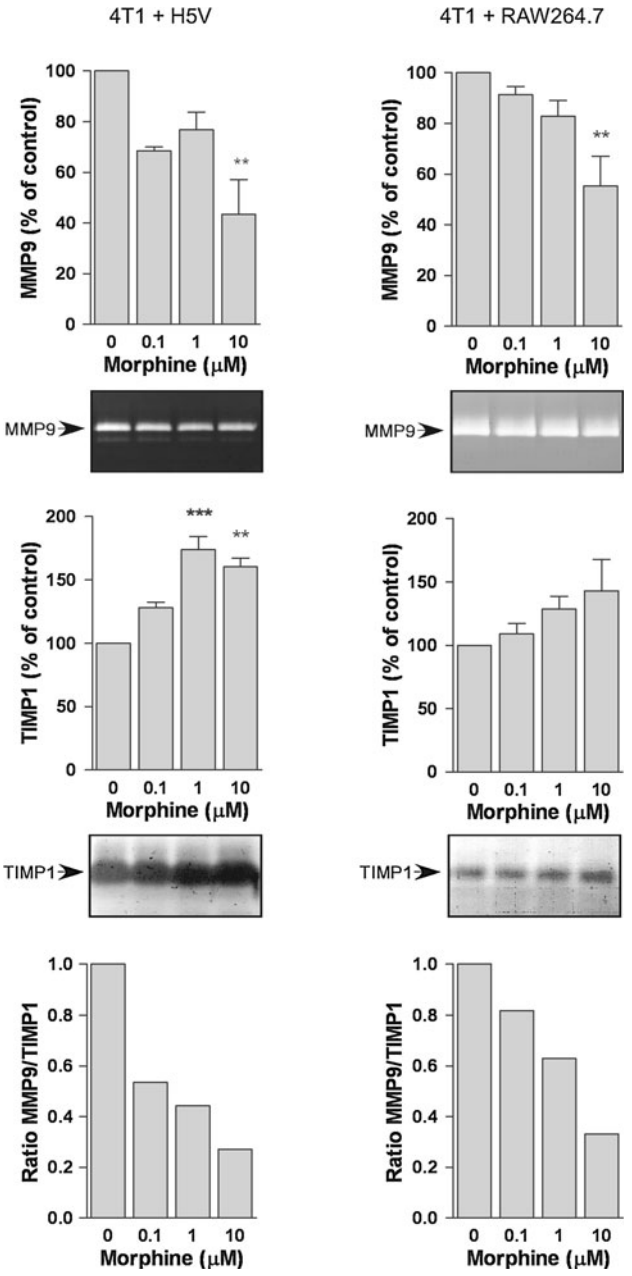

Fig. 5 Morphine reduces MMP-9 and increases TIMP-1 in co-cultured breast cancer and stromal cells. Morphine (up to 10 μM) was applied to co-cultures of 4T1 cells with either H5V or RAW264.7 as indicated. Conditioned media were tested by gelatine or reverse gelatine zymography. Results ($n \geq 3$ separate experiments) were quantified by densitometry. Mean ± SEM is shown, $n = 3$ per group $^{**}p < 0.01$, $^{***}p < 0.001$. The ratio MMP-9/TIMP-1 was further plotted

than when the cells were in contact. This might be due to the fact that overall the conditioned medium is used for a total of four days on live cells (48 h on each cell type) in the assay (Fig. 6) Versus 48 h in total when the cells were in contact (Fig. 5). Alternatively, this unidirectional paracrine effect may only be part of the mechanism by which morphine acts on macrophage-4T1 communication, and this may be enhanced by response from the breast cancer cells or by cell–cell contact.

Other studies have documented an effect of morphine on ECM-degrading enzymes produced by cancer cells. Direct exposure of colon cancer cells to morphine reduced the activity of MMP-2 and MMP-9 in the conditioned media in a concentration-dependent manner [35]. Treatment of HT-29 colon cancer cells with morphine increased uPA production [36]. Similarly Gach et al. showed that morphine decreased MMP-2 and MMP-9 but increased uPA in MCF7 breast cancer cells [37, 38]. Interestingly, when we added morphine to cells grown individually or together, morphine altered the proteolytic profile of co-cultured but not individual cells. These observations are novel and suggest that morphine interferes with the action of paracrine factors or with cross talk between cell types, and further exemplify morphine's different effects between simple and complex systems. We have verified that each of the cell type used in our co cultures, namely 4T1, RAW264.7 macrophages and H5V, express the μ opioid receptor at a level detected via immunoblotting (not shown). Candidate mediators of breast cancer and immune cell interaction, including nitric oxide, colony stimulating factor interleukin-1, interferon-γ, and tumor necrosis factor-α [39–41] can be modulated by morphine administration (reviewed in [2]) although for these factors the effect of morphine has not been demonstrated directly in co-culture systems.

It is difficult to determine whether, in vivo, morphine reduces the proteolytic potential of the tumor microenvironment which in turn reduces tumor growth, or whether morphine otherwise decreases tumor growth, which results in less circulating proteases. The results from our co-culture experiments are in favor of the reduced MMP-9/TIMP-1 ratio as a mechanism for decreased tumor growth. However the co-culture experiments did not show any reduction of uPA by morphine. The in vivo reduction of circulating uPA that we detect in morphine-treated, tumor-bearing mice might thus be secondary to the reduction in MMP-9, the lower number of tumors, or both.

It is obvious from the current literature that morphine acts on tumor growth and metastasis via a myriad of interrelated mechanisms. A conclusively demonstrated, net effect of morphine in cancer surgery patients is still lacking, but clinical trials will hopefully provide answers in the future. In the mean-time, studies like this one contribute to our current knowledge of morphine's effects on breast cancer by dissecting out one such mechanism. Here we showed that while morphine had no effect on the level of ECM proteases produced by cells alone, it could attenuate the potentially pro-invasive paracrine interaction between tumor cells and macrophages or endothelial cells. Our

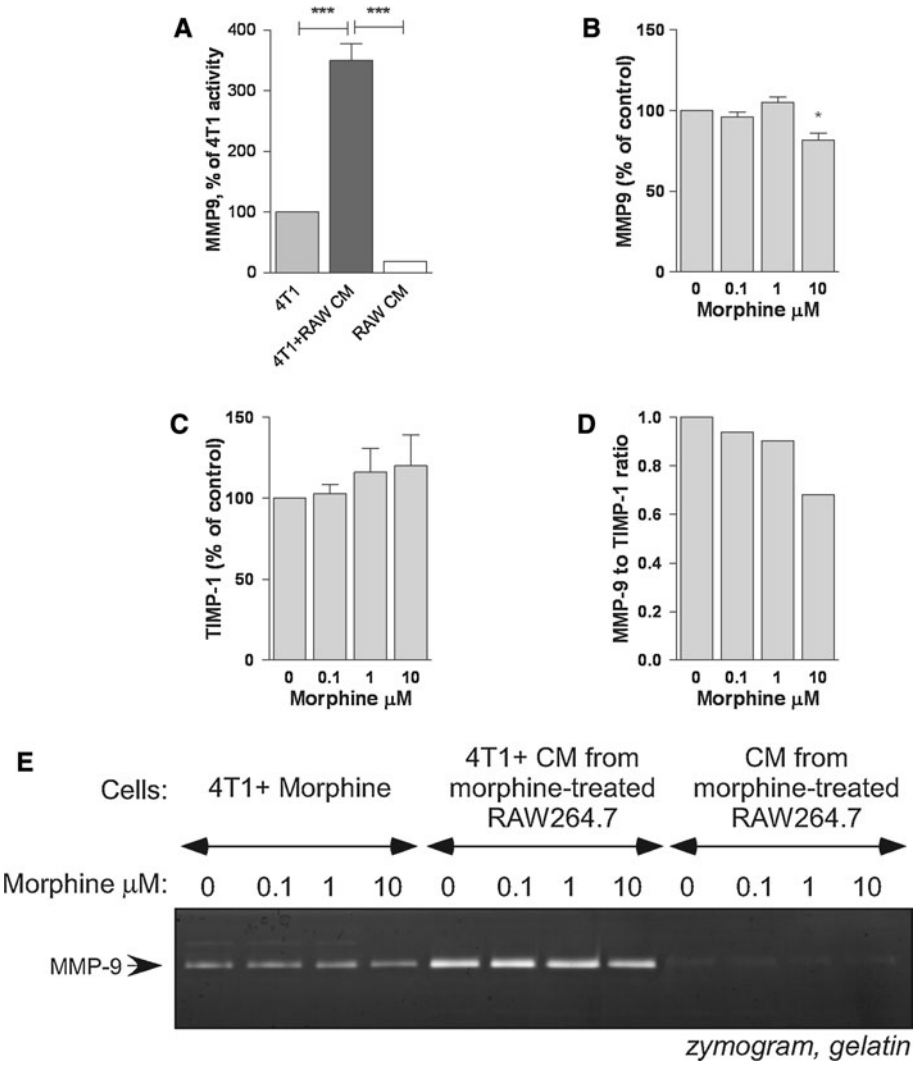

Fig. 6 Morphine modulates the paracrine action of macrophages on breast cancer cell proteolytic profile. **a** 48 h conditioned medium from RAW cells was prepared, then applied to 4T1 cells for 48 h, collected and analysed by gelatin zymography. **b–d** The 48 h conditioned medium from morphine-treated macrophages was applied to 4T1 cells for a further 48 h period, then analysed by gelatine zymography and reverse zymography. **e** Representative zymograph showing the effect of morphine on paracrine cell interaction. Mean ± SEM is shown, n = 3 per group
$^{*}p < 0.05$, $^{***}p < 0.001$

findings indicate a possible new level of anti-tumor effects for morphine in the tumor microenvironment.

References

1. Afsharimani B, Cabot PJ, Parat MO (2011) Morphine use in cancer surgery. Front Pharmacol 2:46
2. Afsharimani B, Cabot P, Parat MO (2011) Morphine and tumor growth and metastasis. Cancer Metastasis Rev 30:225–238
3. Gach K, Wyrebska A, Fichna J, Janecka A (2011) The role of morphine in regulation of cancer cell growth. Naunyn Schmiedebergs Arch Pharmacol 384:221–230
4. Eckhardt BL, Francis PA, Parker BS, Anderson RL (2012) Strategies for the discovery and development of therapies for metastatic breast cancer. Nat Rev Drug Discov 11:479–497
5. Kest B, Palmese CA, Hopkins E, Adler M, Juni A (2001) Assessment of acute and chronic morphine dependence in male and female mice. Pharmacol Biochem Behav 70:149–156
6. Liang DY, Guo T, Liao G, Kingery WS, Peltz G, Clark JD (2006) Chronic pain and genetic background interact and influence opioid analgesia, tolerance, and physical dependence. Pain 121:232–240
7. Ognibene E, Adriani W, Granstrem O, Pieretti S, Laviola G (2007) Impulsivity–anxiety-related behavior and profiles of morphine-induced analgesia in heterozygous reeler mice. Brain Res 1131:173–180
8. Handal M, Grung M, Skurtveit S, Ripel A, Morland J (2002) Pharmacokinetic differences of morphine and morphine-glucuronides are reflected in locomotor activity. Pharmacol Biochem Behav 73:883–892
9. Diaz SL, Hermida MP, Joannas LD, Olivera M, Ridolfi A, Villaamil EC, Balerio GN (2007) Pharmacokinetic aspects of naloxone-precipitated morphine withdrawal in male and female prepubertal mice. Biopharm Drug Dispos 28:283–289
10. Page GG, Ben-Eliyahu S, Yirmiya R, Liebeskind JC (1993) Morphine attenuates surgery-induced enhancement of metastatic colonization in rats. Pain 54:21–28
11. Farooqui M, Li Y, Rogers T, Poonawala T, Griffin RJ, Song CW, Gupta K (2007) COX-2 inhibitor celecoxib prevents chronic morphine-induced promotion of angiogenesis, tumour growth, metastasis and mortality, without compromising analgesia. Br J Cancer 97:1523–1531
12. Shanahan H, Parat MO, Buggy DJ (2013) Could opioids affect cancer recurrence or metastases? Current experimental and

Translational Evidence. In: Parat MO (ed) Morphine and metastasis. Springer, Dordrecht, pp 79–94
13. Gupta K, Kshirsagar S, Chang L, Schwartz R, Law PY, Yee D, Hebbel RP (2002) Morphine stimulates angiogenesis by activating proangiogenic and survival-promoting signaling and promotes breast tumor growth. Cancer Res 62:4491–4498
14. Ishikawa M, Tanno K, Kamo A, Takayanagi Y, Sasaki K (1993) Enhancement of tumor growth by morphine and its possible mechanism in mice. Biol Pharm Bull 16:762–766
15. Yeager MP, Colacchio TA (1991) Effect of morphine on growth of metastatic colon cancer in vivo. Arch Surg 126:454–456
16. Lennon FE, Singleton PA (2013) Opioid regulation of vascular integrity. In: Parat MO (ed) Morphine and metastasis. Springer, Dordrecht, pp 47–61
17. Koodie L, Ramakrishnan S, Roy S (2010) Morphine suppresses tumor angiogenesis through a HIF-1alpha/p38MAPK pathway. Am J Pathol 177:984–997
18. Sasamura T, Nakamura S, Iida Y, Fujii H, Murata J, Saiki I, Nojima H, Kuraishi Y (2002) Morphine analgesia suppresses tumor growth and metastasis in a mouse model of cancer pain produced by orthotopic tumor inoculation. Eur J Pharmacol 441:185–191
19. Parat MO (2013) Morphine and metastasis: from bench to bedside. In: Parat MO (ed) Morphine and metastasis. Springer, Dordrecht, pp 1–13
20. Ustun F, Durmus-Altun G, Altaner S, Tuncbilek N, Uzal C, Berkarda S (2011) Evaluation of morphine effect on tumour angiogenesis in mouse breast tumour model. EATC, Med Oncol 28(4):1264–1272
21. Page GG, McDonald JS, Ben-Eliyahu S (1998) Pre-operative versus postoperative administration of morphine: impact on the neuroendocrine, behavioural, and metastatic-enhancing effects of surgery. Br J Anaesth 81:216–223
22. Hakim TS, Grunstein MM, Michel RP (1992) Opiate action in the pulmonary circulation. Pulm Pharmacol 5:159–165
23. Kim EJ, Shin M, Park H, Hong JE, Shin HK, Kim J, Kwon DY, Park JH (2009) Oral administration of 3,3′-diindolylmethane inhibits lung metastasis of 4T1 murine mammary carcinoma cells in BALB/c mice. J Nutr 139:2373–2379
24. Colombi M, Rebessi L, Boiocchi M, Barlati S (1986) Relationship between circulating plasminogen activators and tumor development in mice. Cancer Res 46:5748–5753
25. Melani C, Sangaletti S, Barazzetta FM, Werb Z, Colombo MP (2007) Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. Cancer Res 67:11438–11446
26. Joyce JA, Pollard JW (2009) Microenvironmental regulation of metastasis. Nat Rev Cancer 9:239–252
27. Hagemann T, Robinson SC, Schulz M, Trumper L, Balkwill FR, Binder C (2004) Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases. Carcinogenesis 25:1543–1549
28. Tjiu JW, Chen JS, Shun CT, Lin SJ, Liao YH, Chu CY, Tsai TF, Chiu HC, Dai YS, Inoue H, Yang PC, Kuo ML, Jee SH (2008) Tumor-associated macrophage-induced invasion and angiogenesis of human basal cell carcinoma cells by cyclooxygenase-2 induction. J Invest Dermatol 129:1016–1025
29. Aoudjit F, Potworowski EF, St-Pierre Y (1998) Bi-directional induction of matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 during T lymphoma/endothelial cell contact: implication of ICAM-1. J Immunol 160:2967–2973
30. Serrati S, Margheri F, Fibbi G, Di Cara G, Minafra L, Pucci-Minafra I, Liotta F, Annunziato F, Pucci M, Del Rosso M (2008) Endothelial cells and normal breast epithelial cells enhance invasion of breast carcinoma cells by CXCR-4-dependent up-regulation of urokinase-type plasminogen activator receptor (uPAR, CD87) expression. J Pathol 214:545–554
31. Kargozaran H, Yuan S, Breslin J, Watson K, Gaudreault N, Breen A, Wu M (2007) A role for endothelial-derived matrix metalloproteinase-2 in breast cancer cell transmigration across the endothelial-basement membrane barrier. Clin Exp Metastasis 24:495–502
32. Neiva KG, Zhang Z, Miyazawa M, Warner KA, Karl E, Nor JE (2009) Cross talk initiated by endothelial cells enhances migration and inhibits anoikis of squamous cell carcinoma cells through STAT3/Akt/ERK signaling. Neoplasia 11:583–593
33. Ingthorsson S, Sigurdsson V, Fridriksdottir A Jr, Jonasson JG, Kjartansson J, Magnusson MK, Gudjonsson T (2010) Endothelial cells stimulate growth of normal and cancerous breast epithelial cells in 3D culture. BMC Res Notes 3:184
34. Hagemann T, Wilson J, Kulbe H, Li NF, Leinster DA, Charles K, Klemm F, Pukrop T, Binder C, Balkwill FR (2005) Macrophages induce invasiveness of epithelial cancer cells via NF-kappa B and JNK. J Immunol 175:1197–1205
35. Harimaya Y, Koizumi K, Andoh T, Nojima H, Kuraishi Y, Saiki I (2002) Potential ability of morphine to inhibit the adhesion, invasion and metastasis of metastatic colon 26-L5 carcinoma cells. Cancer Lett 187:121–127
36. Nylund G, Pettersson A, Bengtsson C, Khorram-Manesh A, Nordgren S, Delbro DS (2008) Functional expression of mu-opioid receptors in the human colon cancer cell line, HT-29, and their localization in human colon. Dig Dis Sci 53:461–466
37. Gach K, Szemraj J, Wyrebska A, Janecka A. The influence of opioids on matrix metalloproteinase-2 and -9 secretion and mRNA levels in MCF-7 breast cancer cell line. Mol Biol Rep 2010
38. Gach K, Szemraj J, Fichna J, Piestrzeniewicz M, Delbro DS, Janecka A (2009) The influence of opioids on urokinase plasminogen activator on protein and mRNA level in MCF-7 breast cancer cell line. Chem Biol Drug Des 74:390–396
39. Lin CW, Shen SC, Ko CH, Lin HY, Chen YC (2010) Reciprocal activation of macrophages and breast carcinoma cells by nitric oxide and colony-stimulating factor-1. Carcinogenesis 31: 2039–2048
40. Sarkar DK, Murugan S, Zhang C, Boyadjieva N (2012) Regulation of cancer progression by +/−endorphin neuron. Cancer Res 72:836–840
41. Kawata M, Koinuma D, Ogami T, Umezawa K, Iwata C, Watabe T, Miyazono K (2012) TGF-+/−-induced epithelial–mesenchymal transition of A549 lung adenocarcinoma cells is enhanced by pro-inflammatory cytokines derived from RAW 264.7 macrophage cells. J Biochem 151:205–216