

Inhibitors for Metastasis Development

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Abstract: A leading cause of death, cancer remains the bane of modern society and one of the most challenging research fields. Cancer is initially a localized disease that can be often treated well at a very early stage. However the vast majority of cancer deaths result from a pernicious progression of the disease, the development of distant metastases. It must therefore be a pressing research goal to focus on the pharmacological prevention of metastasis development. This review summarizes the current understanding of the cellular and molecular mechanisms of metastasis development, and suggests possible approaches for its inhibition.

Keywords: Cancer, metastasis, migration, hormones, chemokines, neoneurogenesis, neurotransmitters, seven-helices receptors.

INTRODUCTION

Cancer research is presently undergoing a fundamental shift with regard to the treatment of the disease. All prophecies made by scientists in the last century that cancer will be curable in the 21st century, have in the light of the status of our war against cancer turned out to be wrong. Although the long-term goal still remains to make this life-threatening disease curable one day, we have to accept at present that our understanding to do so is yet not comprehensive enough. The short-term goal is thus to turn the deadly disease into a chronic one: To elongate the survival time of the cancer patients with a maximum of life quality. In order to reach this short-term goal, two features of cancer have to be taken into account. First, the growth of the tumor must not exceed a certain size - so that the according organ in which the tumor is located is not seriously affected in its function. Second, the spreading of the tumor and the development of (micro-) metastases must be inhibited. Thus, growth and metastasis development are the critical parameters. We will herein present and discuss classes of anti-cancer drugs with regard to metastasis inhibition. Furthermore, we will point out that a number of drugs which are termed as metastasis inhibitors do not actually inhibit cell functions, which are essential for metastasis development, e.g. migratory activity or extravasation, but are substances of other spectra of efficacy, e.g. proliferation inhibition.

The growth of a tumor has been under more intensive investigation than the mechanisms of metastasis development have been to date. Consequently, more pharmaceuticals are available which target tumor growth inhibition. There are at the moment two major functional classes of drugs established for the inhibition of tumor growth. The first group consists of proliferation inhibitors, the second group consists of angiogenesis inhibitors. The proliferation inhibitors directly inhibit the cell cycle and the mitosis of the tumor cells, or they use the high proliferative

activity and the concomitant higher susceptibility for replication errors, which cause lethal alterations of the genome. From the type of action, we distinguish three classes of proliferation inhibitors which directly act on the DNA: first, the alkylating agents. These substances constitute the first class of agents that have been used in chemotherapy. These drugs, e.g. melphalan, carboplatin, and cisplatin [1], modify the bases of DNA, interfering with DNA replication and transcription. The second class are the intercalating agents, such as adriamycin or doxorubicine (anthracyclines), which interfere with transcription replication similar to the alkylating agents [2]. Third, the enzyme inhibitors: These drugs inhibit key enzymes, such as topoisomerases [3], which are involved in DNA replication. Their inhibition induces DNA damage or inhibits the repair of damage during replication. Among the second class - the intercalating substances - are pharmaceuticals which are also applied as patents for the inhibition of metastases. These are carbocyclic phosphatidic acid derivatives [4], (bis)cyclopropanecarboxylic acid amides [5, 6], and benzene as well as benzoic acid derivatives [7, 8]. However, as mentioned above, these substances do not inhibit the metastasis process *per se*, but the outgrowth of the metastasizing cells to a secondary tumor. It might well be that the metastasizing cells survive the treatment with growth inhibitors as non-proliferative dormant cells [9].

Besides these substances, which directly act on the DNA, other substances have been established for the inhibition of tumor growth, which act on proteins of the mitotic machinery or on signaling pathways regulating the cell cycle. For example, paclitaxel stabilizes the microtubules [10], and thus prevents the organization of the mitotic spindle, which is essential for the cell division. In consequence, the cells are arrested in the late G₂- and M-phase of the cell cycle. In addition, although there is no proof for a direct involvement of microtubules in the generation of locomotor forces in tumor cell migration, this cell function might also be affected by an increased stiffness of the cell through the stabilization of the microtubules. Paclitaxel has been approved for the treatment of ovarian cancer in 1992. Raltitrexed is another substance which acts on a protein associated with the mitotic

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machinery. This drug inhibits the thymidylate synthase, which leads to the depletion of deoxythymidine triphosphate (dTTP). Since dTTP is one of the four components for the synthesis of DNA, its synthesis is inhibited [11]. Metabolites of 5-fluoro-uracil (5-FU), one of the oldest chemotherapeutic agents, have a function similar to Raltitrexed. In addition, 5-FU is incorporated into the DNA and RNA and inhibits chain-elongation and mRNA conversion, respectively [11]. Because of their lack of specificity, these agents have debilitating and broad side effects, which often limit their usage. However, despite these side effects, uracil derivatives are widely used and there are patents applied for metastasis inhibition [12], too.

Cattley and Radinsky have recently reviewed a group of therapeutics, which they termed as therapies against self-sufficiency in growth-signals [13], according to one of the six hallmarks of cancer postulated by Hanahan and Weinberg [14]. HER2 (erbB2) is a tyrosine receptor kinase (RTK) of the epidermal growth factor (EGF) receptor class. This kinase is frequently overexpressed in breast cancer, and its expression is correlated with a poor prognosis [15]. The activation of the RTKs of the EGFR family leads to an increased proliferation of normal epithelial cells [16] and tumor cells [17]. Gefitinib is a specific inhibitor for these RTKs, and has been approved for the treatment of non-small cell lung cancer [18]. An overexpression of HER2 increases the sensitivity of non-small cell lung cancer cells to this drug [19]. Likewise, an inhibitor for the insulin-like growth factor (IGF) -I and -II is an applied patent for metastasis inhibition [20]. A blockade of the binding of these ligands to their RTKs might provide a promising tool for the inhibition of metastases, since it is known that IGF-I and -II regulate the migration and integrin-mediated adhesion of carcinoma cells of different tissues [21-24]. Another class of tyrosine kinase inhibitors used in the anti-cancer chemotherapy is constituted by inhibitors for BCR-Abl. BCR-Abl is a non-receptor tyrosine kinase resulting from a chromosomal translocation known as the Philadelphia chromosome [25]. Imatinib mesylate is an inhibitor for this tyrosine kinase and is used in the therapy of chronic myeloid leukemia [26].

The second group of growth inhibitors, the angiogenesis inhibitors, prohibit the formation of blood vessels in tumor tissues. Sustained angiogenesis is important for the growth of a tumor [26]. Without such generation of new blood vessels, a tumor can not grow over a certain size due to the lack of nutrition. A very important cytokine with regard to angiogenesis is the vascular endothelial growth factor (VEGF). VEGF is secreted by tumor cells in response to cellular stress, induced by hypoxia [27]. VEGF binds to the VEGF receptor II on endothelial cells and supports their proliferation and survival [28, 29]. Approaches to inhibit angiogenesis are made by two ways. First, VEGF is neutralized by an anti-VEGF antibody [30]. Second, the tyrosine kinase activity of the VEGF receptor II is inhibited [31]. Although these are promising approaches, the regulation of angiogenesis depends not merely on VEGF, but is a balanced process of several angiogenic and angiostatic factors. For example, Strieter and his group have elaborated on the role of CXC chemokines in this balance, showing the complexity of the regulation of angiogenesis [32]. In light of this multitude of involved regulators it might be difficult to

efficiently and definitely inhibit angiogenesis on this site. Another, more direct approach is provided by fumagillol derivatives, which are inhibitors of endothelial cell proliferation [33], and are applied as patents for metastasis inhibition [34].

In contrast to the aforementioned drug developments which target tumor growth, the inhibition of metastasis development and drugs specifically targeting this tumor promotion step were for a long time almost disregarded. This review will thus focus on current theories on how the development of metastasis takes place and how strategies can be developed to inhibit the spreading of the tumor over the entire organism.

GENOMIC AND EPIGENETIC – TWO ASPECTS OF CANCER AND METASTASIS DEVELOPMENT

We are said to be in the post-genomic era of cancer research. What does this mean? Scientists have put much effort into the discovery of the genetic basis of carcinogenesis and tumor progression, and they have delivered a valuable basis for present research. The function of the identified genes has to be unraveled and to be set into the context of cell function and interaction with the environment. With regard to metastasis development the term 'post-genomic era' has a somehow deeper meaning. There was a long-standing theory that the development of metastases is genetically based. This theory is now supplemented or maybe even opposed by a new theory that environmental factors lead to the onset of the metastasis cascade.

In 1990 Fearon and Vogelstein published a sequential model for the transition of normal colon epithelium to colorectal carcinoma, showing discrete genetic events as components of this transition [35]. Accordingly, Hahn *et al.* showed that they were able to generate a cell type with tumor behaviour with a very limited number of genetic alterations inserted into normal epithelial cells and fibroblasts [36]. These mutations were the simian virus 40 large-T oncoprotein, an oncogenic allele of H-ras and an active telomerase (hTERT), and were thus very much in accordance with Fearon and Vogelstein's findings. However, no genetic model has been able to adequately describe the events that lead to metastasis development. Bernards and Weinberg propose that mutant genes confer a darwinian selective advantage for a clonal selection of metastatic tumor cells [37], and Hanahan and Weinberg accordingly suggest tissue invasion and metastasis as one of six genotypic manifestations of cancer [14]. However, Weigelt *et al.* have shown by comparative gene expression profiling striking similarity between the primary breast tumors and the distant metastases, suggesting that the metastatic capability in breast cancer is an inherent feature and is not based on clonal selection [38]. Thus, although several proteins have been described to be associated with a higher metastatic potential, e.g. the metastasis-associated protein 1 [39], no gene regulation has been identified to be the causative link to metastasis development. So how can the onset of metastasis development and the localization of metastases be explained? The general critique on the genetic view of metastasis development is its reductionary component. Therein tumor cells are examined in an isolated fashion to the exclusion of environmental influences. *In vivo*, a tumor is

always embedded within the tissue it originates from, and thus might still be subject to the same or at least similar regulations. The neuro-endocrine system represents a superordinate regulatory element within the organism. It is not independent from other systems in terms of crosswise regulation, but rather the release of neurotransmitters and hormones in response to certain influences (including sensations and emotions) serves for a coordinated regulation of organs. The crucial characteristic in tumor cells is the disbalance of regulation. Due to their de-differentiation, the cells might lose their ability to respond to a certain signal and gain the ability to recognize ligands, for which the normal tissue previously had no receptors.

One of the best known examples of a dysregulated function of hormones is the role of estrogens in breast cancer. In premenopausal women, the ovaries are the main source of estrogen synthesis, in postmenopausal women estrogens are synthesized by adipose tissue. Obviously, the exposure time to estrogen plays a central role in breast cancer development, as an early menarche and late menopause increase the cancer risk [40]. Furthermore, the use of oral contraceptive [41], or postmenopausal hormone replacement therapy [42] have been implicated in an increased cancer risk, too. However, this remains a subject of current controversy. While estrogen has a vital physiological function in female development and pregnancy, estrogens can also be regarded as carcinogenics depending on their structure and dose [43]. Estrogen promotes the growth and development of the normal mammary gland and likewise promotes the proliferation of breast cancer cells [44, 45]. In addition, the metabolites of estrogen, the catechol-estrogen-3,4-quinones, react with the DNA to form predominantly depurinating adducts [46], which produce replication errors and mutations. As stated above, the main source of estrogens in postmenopausal women is adipose tissue. Here, estrogens are synthesized from androstenedione (which is released by the adrenal gland) by the enzyme aromatase [47]. Consequently, inhibitors for the aromatase have been developed, which are divided into steroidal (formestane, exemestane) and non-steroidal (letrozole, anastrozole) drugs [48]. Yue *et al.* discuss an advantage of these inhibitors in breast cancer therapy in comparison to estrogen receptor blockers such as tamoxifen or toremifene, since the aromatase inhibitors prevent not only the direct receptor-mediated effect of estrogen, but also the above discussed depurinating effect of its metabolites [49, 50]. In contrast, it is noteworthy that estrogens reduce the risk of colon cancer [51, 52].

Similar to cancer of the breast, the tumor growth of prostate cancer depends on the male hormone testosterone [53]. Hormonal deprivation therapy is the standard therapy for advanced (stage IV) prostate cancer [54]. The two organs in the human body that release androgens are the testicles and the adrenal glands. The release of androgen by the testicles can be blocked by luteinizing hormone-releasing hormone (LHRH) agonists [55] such as goserelin or leuprorelin [56]. Furthermore, androgen function can be blocked directly by anti-androgen drugs, such as the non-steroidal anti-androgens flutamide and casodex [53].

These examples show that a tumor, similar to its normal tissue of origin, is subject to regulatory influences from the environment. Especially the neuro-endocrine system constitutes a superordinate instance in the body, which regulates the function and homeostasis of organs and tissues.

POSITIVE AND NEGATIVE REGULATION OF TUMOR CELL MIGRATION

While hormones - especially steroid hormones - have a direct gene regulatory effect and usually lead to long-term changes of gene regulation, neurotransmitters initiate several pathways that have immediate effects on cell functions. However, neurotransmitters lead to gene regulation, too [57], and hormones can also have short-term effects, e.g. on the migration of leukocytes [58]. This shows that these groups of signal substances have a functional intersection. In contrast to steroid hormones (which bind to intracellular receptors), neurotransmitters bind to membrane receptors of the seven-helices family. Ligands to these receptors, i.e. chemokines and neurotransmitters, are probably the most important regulators of cell migration. Engagement of seven-helices receptors by these ligands regulates the migration of leukocytes and tumor cells [59, 60].

Chemokines are peptides, which are subdivided into four families due to the relative position of two aminoterminal cysteins. The CC chemokines and CXC chemokines constitute the two largest families. Here, the two cysteins are directly adjacent (CC) or separated by one amino acid (CXC), respectively [61]. The two other families are the XC or C chemokines, in which the first amino acid is missing, and the CX3C chemokines with three separating amino acids between the cysteins. About 50 chemokines and 20 chemokine receptors are known to date [62]. This proportion between ligands and receptors suggests already that more than one chemokine binds to a certain receptor. Surprisingly, the chemokine system is redundant even in the other direction, as one ligand can bind to several receptors.

Chemokines are involved in the regulation of tumor cell functions including growth, angiogenesis (as already described above), survival and migration [63]. Furthermore, functional similarities between the processes in cancer development and inflammation have been described by Balkwill and Mantovani [64]. A schematic overview of chemokines with a potential role in metastasis development is given in Tab. (1). The best investigated chemokine with regard to its function in tumor cell migration is the stromal cell-derived factor-1 (SDF-1), also termed as CXCL12. Mueller *et al.* showed that several human cell lines of breast cancer and melanoma express the SDF-1 receptor CXCR4, besides other chemokine receptors such as CXCR2, CCR7, CCR8 and CCR10 [65]. They used the CXCR4-expressing breast cancer cell line MDA-MB-231 in a mouse model to show that this cell line has a high tendency to migrate to organ destinations that express high amounts of SDF-1. Furthermore, treatment of the mice with a CXCR4 neutralizing antibody impaired the metastasis development in regional lymph nodes and the lung [65]. Helbig *et al.* describe that the transcription factor NF- κ B upregulates the expression of CXCR4 and refer to a constitutive activation of NF- κ B in a variety of cancers [66]. Therefore, the question is, how is NF- κ B activated? Lounikova *et al.* showed that

Table 1. Overview of Chemokines and Neurotransmitters with a Regulatory Function on Tumor Cell Migration and Metastasis Development

CHEMOKINES	Ligand	Receptor	References
CC	6Ckine	CCR7	a
CXC	SDF-1 (CXCL12)	CXCR4	a
	MIG (CXCL9)	CXCR3	b
	IP-10 (CXCR3)	CXCR3	b
	Interleukin-8 (CXCL8)	CXCR1/2	c
CX3C	Fractalkine (CX3CL1)	CX3CR1	d
NEUROTRANSMITTERS	Ligand	Receptor	References
Stress-related	Catecholamines	Beta2-AR	e, f
	Dopamine	D2R	e
	Substance P	NK-1R	e, g
Opioid peptides	Met-enkephalin	DOR	h
	Beta-endorphin	DOR	i
Inflammatory	Histamine	H1R	j
	Bradykinin	B1R	k
Gastrointestinal	Bombesin/GRP		l, m
	Gastrin/CCK	CCK-A/BR	n
	VIP		l, o
	Endothelin		p
Inhibitory	GABA	GABA-BR	q
	Anandamide	CB-1R	r

a = ref. 65, b = ref. 73, c = ref. 75, d = ref. 74, e = ref. 57, f = refs. 95, 96, g = ref. 60, h = ref. 120, i = ref. 108, j = ref. 125, k = ref. 129, l = ref. 136, m = ref. 137, n = refs. 142, 143, o = ref. 146, p = ref. 152, q = 155, r = refs. 160, 161.

several cytokines, including EGF, activate NF- κ B in murine squamous cell carcinoma, which led to an enhanced expression of the chemokine Gro1 [67]. Thus, environmental factors are able to induce receptor or ligand expression, which in turn sensitize the cell for other signals. Meanwhile, the role of SDF-1 in metastasis development has been confirmed for prostate carcinoma cells [68, 69], as well as a role of this chemokine in the growth of ovarian carcinoma cells [70] and in the adhesion of head and neck squamous carcinoma cells [71]. The SDF-1 receptor CXCR4 is expressed on several cancers of epithelial, mesenchymal and haematopoietic origin [72]. Besides SDF-1/CXCL12, Mueller *et al.* found that the 6Ckine/CCL21 induced directional migration of breast carcinoma cells similar to SDF-1/CXCL12, but they did not perform animal experiments with respect to this chemokine [65]. A second animal study with a CXC chemokine receptor was conducted by Kawada *et al.*, showing that the development of lymph node metastases depends on the engagement of CXCR3 by its appropriate ligands CXCL9/MIG and CXCL10/IP-10 [73].

In addition, *in vitro* studies have shown that the fractalkine receptor CX3CR1 is expressed on prostate cancer cells, and bone marrow endothelial cells as well as differentiated osteoblasts express the according ligand fractalkine/CX3CL1 [74]. Fractalkine is the only membrane-bound chemokine, and a neutralizing antibody to fractalkine thus reduces the adhesion of prostate cancer cells to the bone marrow cells [74], which suggests a role of this chemokine in the metastasis development of prostate cancer. Furthermore, bladder epithelial cells express the inflammatory chemokine interleukin-8 and release it in response to bacterial infection in order to attract immune cells, especially neutrophil granulocytes. The bladder cancer cell line T24 constitutively releases interleukin-8 and expresses the according interleukin-8 receptor A (IL-8RA) [75]. By this mechanism, the T24 cells increase their migratory and proliferative activity in an autocrine fashion. Taken together, these results on the involvement of the chemokine system in tumor cell migration not only support the view of Balkwill and Mantovani, that inflammation may contribute to tumor progression, but furthermore support

their idea of a potential use of chemokine receptor blockers for the inhibition of metastasis development [64]. Chemokine receptor antagonists are under development for diseases such as the CXCR4 antagonist KRH-1636 in HIV [76], or CCR1 antagonists like CP-491,715 in rheumatoid arthritis [77, 78] and in multiple sclerosis [79]. Furthermore, patents are applied for a non-peptide CCR1 receptor for the treatment of renal fibrosis [80], for an aminoterminal extended RANTES [81] as well as for piperidine compounds [82] blocking the CCR3, for an antibody blocking the CCR4 [83], for pyrrolidine compounds modulating the CCR5 [84], and for pyrimidine derivatives blocking the fractalkine receptor CX3CR1 [85]. With regard to the inhibition of metastasis development, CXCR4 antagonists have been applied for patents [86, 87]. Further examples for chemokine receptor antagonists are presented in a recent review by Chen and colleagues [88]. However, a problem with the use of these antagonists might arise from the above discussed redundancy of the chemokine system. The blockade of a certain receptor or neutralization of a certain chemokine might be functionally substituted, or can cause severe adverse effects. Interestingly, in some cases this redundancy may turn out as an advantage. For example, persons with a mutant CCR5 are resistant to HIV infection, but have no obvious impairment of the immune system, which might be ascribed to the substitution of the CCR5 by other chemokine receptors.

In the context of the above described interconnection between inflammatory mediators and cancer as introduced by Balkwill and Mantovani [64], it is noteworthy that olibanum now comes back into focus in cancer research. Olibanum (incense) has been used as a remedy since ancient times. Nowadays, extracts of olibanum, the boswellic acids, have been identified as potent anti-inflammatory compounds and are used in the treatment of chronic inflammatory diseases [89]. Furthermore, boswellic acid has anti-cancer effects [90-92], and boswellic acid has been applied for patent by a Chinese group [93].

In the neurotransmitter system, the relation between the number of ligands and receptors is opposite to the chemokine system and the binding is more restricted, less redundant. Here, several receptors are present, which are specific for one neurotransmitter. The expression of the receptors varies among tissues and it is thus possible to inhibit the effect of a neurotransmitter in a certain tissue without affecting its function in another tissue. Table (1) summarizes the current knowledge of neurotransmitters with a function in tumor cell migration and the engaged receptor, where applicable.

Stress is one of the strongest and most important reactions in the animal kingdom (fight-or-flight reaction). Catecholamines - epinephrine and norepinephrine - are the neurotransmitters mediating this stress reaction and therefore are also known as stress hormones. They induce an increase of the intensity and frequency of heart muscle contraction, dilation of airways and an increase of the blood glucose concentration. Stress is a major risk factor, not only for a cardiovascular disease, but also for the development of cancer. Psychosocial factors like job-related stress have been implicated in tumor progression as early as 1926 [94]. Catecholamines are derivatives of the amino acid tyrosine.

Epinephrine and norepinephrine represent the prominent representatives in vertebrates. Norepinephrine induces the migration of carcinoma cells of various tissues by the engagement of 2-adrenoceptors [57, 95, 96], and epinephrine modulates the carcinogenesis in the lung of hamsters induced by the tobacco-specific nitrosamines [97]. The 2-adrenoceptor is expressed in the tumors of several tissues, as shown in Fig. (1A). Furthermore, norepinephrine induces chemotaxis in tumor cells [95], which strongly supports a functional involvement of this neurotransmitter in the development of metastases similar to chemokines. Furthermore, noradrenergic nerve fibers are found in the spleen, thymus, bone marrow, and lymph nodes [98], which may provide a chemoattractive source for tumor cells, as we and others have postulated previously [99, 100]. At present, there is to our knowledge no specific 2-adrenoceptor antagonist approved for the treatment of cancer, especially for the inhibition of metastasis development. However, subtype-unspecific 1/2-adrenoceptor blockers have been used for the treatment of cardiovascular diseases before they were displaced by 1-specific drugs. These subtype-unspecific antagonists might be interesting pharmaceuticals for the inhibition of catecholamine-driven metastasis development as long as no 2-specific antagonist is available.

Dopamine is the precursor in the synthesis of catecholamines and is also released in a stress reaction. Furthermore, dopamine has been implicated in the pathological conditions of schizophrenia and Parkinson's disease [101]. Dopamine elicits a promigratory effect in breast carcinoma cells with a potential similar to norepinephrine [57]. This effect is mediated by the dopamine D2 receptor (D2R), as we have shown by the use of the specific receptor blocker metoclopramide [57]. The D2R is also expressed in tumors of several different tissues, and it has a striking similarity to the tissue expression pattern of the 2-adrenoceptor; Fig. (1B). Metoclopramide is a well established drug for the treatment of nausea and vomiting, and might - with the accompanying extension of statutory approval - also be an effective tool for the inhibition of dopamine-mediated tumor progression.

Substance P is a peptide of the neurokinin family, localized in the peripheral nervous systems and in regions of the central nervous system which coordinate stress reactions [102, 103]. Substance P plays a role in the regulation of affective behaviour, anxiety and depression, and a pharmacological inhibition of the neurokinin (NK)-1 receptor, the preferential receptor for substance P, is an effective tool for the treatment of depressive disorders [104]. With regard to cell migration, substance P might contribute to the pathogenesis of asthma, because of its inflammatory effects on the airways [105]. Substance P influences the migration of neutrophil granulocytes across endothelial and subendothelial barriers towards inflammatory sites of the lung, thereby regulating their interstitial accumulation and traffic to the alveolar space [106, 107]. The neurokinin-1 receptor is expressed in several tumor tissues. However, the expression is distinct from the expression of the 2-adrenoceptor and dopamine D2 receptor; Fig. (1C). Substance P induces the migration of colon [60] and breast [57] carcinoma cells, and is a chemoattractant for small cell

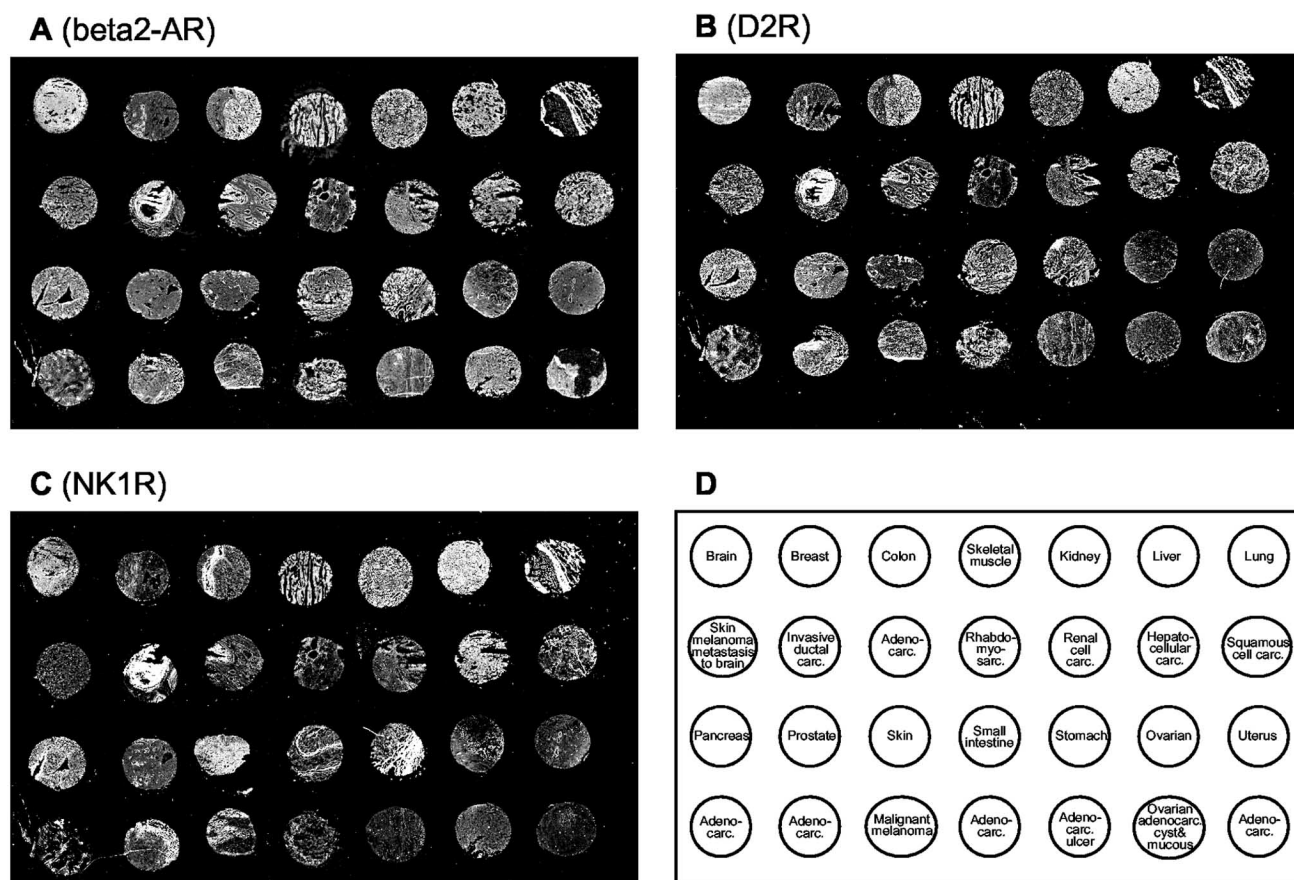


Fig. (1). Tissue microarrays of normal tissues and tumors of the respective tissue. First and third lane: normal tissue; second and fourth lane: tumors of the same type of tissue as the normal tissue above. Microarrays were provided by BioCat (Heidelberg, Germany) and stained with an anti- β_2 -adrenoceptor antibody (A), an anti-dopamine D2 receptor antibody (B), or an anti-neurokinin-1 receptor antibody (C). In (D), the respective tissue origin is shown. The sections were treated with 0.3% H_2O_2 for 30 minutes at room temperature. After washing with PBS, the sections were blocked with 10% normal bovine serum for one hour at room temperature, and subsequently incubated with the primary antibodies at 4°C overnight. After a further washing step with PBS, the sections were incubated with a Cy3-coupled secondary antibody for one hour at room temperature. After intensive washing with PBS, the fluorescence of the samples was analyzed using a dual-laser microarray scanner at 532 nm.

lung carcinoma cells [108]. Thus, blockade of the involved NK-1 receptor can potentially inhibit metastasis development which is induced by this neurotransmitter. A Japanese group has introduced such an antagonist, however, as an analgetic drug [109].

Opioid peptides rank functionally on the opposite side of substance P in the spectrum of affective behaviour [110], and furthermore play a role in stress-defense [111] and analgesia [112]. Proopiomelanocortin (POMC) is the neuropeptide precursor for the opioid peptides β -endorphin, enkephalins and dynorphins. The active neuropeptides are generated from this molecule by post-translational cleavage. The POMC gene is not only expressed by cells of the nervous system, but also by leukocytes [113, 114]. Thus, the immune system also regulates the control of pain [115, 116]. Immune cells release the opioid peptides in inflamed tissue, which act on opioid receptors of peripheral sensory nerve endings [117]. Opioid peptides thus counteract proinflammatory cytokines such as interleukin-1 and -6, which are mediators of exaggerated pain [118]. These cytokines activate pain-

responsive sensory nerve terminals, which leads to the release of substance P [119]. Substance P in turn has proinflammatory effects, too, and forms a positive feedback loop with the proinflammatory cytokines [118]. With regard to the regulation of tumor cell migration by neurotransmitters, we have shown that met-enkephalin induces migration in breast carcinoma cells [120], whereas β -endorphin has no effect [95]. Met-enkephalin binds to the β -opioid receptor (β -OR or DOR), and with ten-fold lower affinity to the μ -opioid receptor (μ -OR or MOR), while β -endorphin is equiactive on both these receptors [121]. The mechanism of regulation is not yet clear, but from experiments with specific receptor agonists we hypothesized that DOR has stimulatory function and MOR inhibitory function. In consequence, the β -endorphin effect is compensated to zero by both receptors, whereas met-enkephalin has a stimulatory net effect through its higher affinity to the DOR [120]. However, in small cell lung carcinoma, β -endorphin has been shown to have a chemoattractant function [108]. Thus, it is likely that tumor cell migration can be regulated by opioid receptors and it is

therefore prudent to investigate agonists and antagonists to these receptors. For example, nociceptin is a ligand to the orphan receptor opioid receptor-like 1 (ORL1) and analogs of nociceptin are applied for patent [122, 123].

As discussed above, Balkwill and Mantovani have woven the link between cancer and inflammation [64]. What role do neurotransmitters play in this interaction? The role of substance P in inflammation has already been discussed above, and there are several other neurotransmitters and hormones, which are involved in inflammatory processes. Histamine is a central nervous neurotransmitter in vertebrates and invertebrates, and is released locally in the inflammatory response by mast cells. It has been reviewed recently by Kavekos that histamine also causes suppression of interferon-induced proteins needed for anti-tumor response and activates T-suppressor cell function in cancers such as squamous cell carcinoma and melanoma [124]. Furthermore, histamine has chemoattractive function on human carcinoma and melanoma cells by engagement of the H1R [125]. These results suggest an anti-histamine approach for the treatment of cancer [124]. In diametral contrast to this, an increased survival rate of patients with metastatic melanoma by the treatment of histamine in combination with interleukin-2 was shown in clinical phase II and III trials [126, 127].

The results on bradykinin, a further inflammatory neurotransmitter, are more consistent than those on histamine. Bradykinin exerts its proinflammatory effect via the activation of the constitutively expressed B2 receptor, whereas the B1 receptor is activated by a biological active metabolite of bradykinin. The B1 receptor is underexpressed in normal tissues and upregulated during inflammatory responses [128]. In human prostate tissue, the B2R is ubiquitously expressed, whereas the B1R is detected only in malignant tissue [129]. Furthermore, stimulation of the B1R promotes growth, migration, and invasion of PC-3 prostate cancer cells [129]. Accordingly, treatment with anti-bradykinins has been suggested as anti-cancer therapy especially for lung and prostate cancers [130].

Inflammation is regarded as a major risk factor for cancers of the gastrointestinal tract [131]. The regulation of gastrointestinal cell function is somewhat more complex than of other tissues, since the gastrointestinal system is subject to not only the general systemic regulation, but is in addition regulated by a largely autonomous neurotransmitter system, which is independent of a superordinate regulation. In the gastrointestinal system, four neuropeptides are known to have a regulatory function in tumor cell migration. Bombesin and its mammalian homologue GRP mediate the release of gastrin and cholecystokinin (CCK) from gastrointestinal endocrine cells of the stomach and upper small bowel, respectively. Both bombesin/GRP and gastrin/CCK exert direct effects on tumor cell migration. Receptors for bombesin/GRP are expressed in tumors from various tissues including the colon [132], prostate [133], breast [134] and lung [135]. Bombesin stimulates the invasive potential and migration of colon and prostate carcinoma cells *in vitro* [136, 137], whereas the identification of the responsible bombesin receptor subtype was not a subject of these studies. Gastrin and CCK bind to two related receptors, which are

differentially expressed in the gastrointestinal tract. The CCK-A receptor is expressed in pancreatic acinar cells, whereas the CCK-B receptor is mainly expressed in the stomach [138]. According to their expression in normal tissues, the CCK-B receptor is expressed in adenocarcinomas of all areas of the gastrointestinal tract, whereas the CCK-A receptor is rarely expressed in gastroenteropancreatic tumors [139-141]. CCK and gastrin promote the invasiveness of human pancreatic cancer cell lines [142], and of human colon cancer cells [143], respectively.

Besides these important gastrointestinal neuropeptides with a function restricted to the gastrointestinal system, two further neurotransmitters have a known effect on tumor cell migration. The first is the vasoactive intestinal polypeptide (VIP), which is expressed not only in gastrointestinal organs, but also in the lung and the prostate [144]. Receptors for VIP are found in tumors of the gastrointestinal tract such as ductal pancreatic, gastric, or colorectal carcinoma, as well as in lung, breast and bladder carcinoma and other types of cancer [145]. Nagakawa and co-workers have shown by the use of various assays and cell lines that VIP increases the invasive potential of prostate carcinoma cells [136, 146].

Endothelin is primarily produced in the endothelium in response to stress mediators such as norepinephrine [147]. It contributes to the regulation of blood pressure by vasoconstriction [148], but is also known to play a role in the regulation of gastrointestinal function [149-151]. Endothelin-1 is overexpressed in Kaposi's sarcoma lesions and promotes invasiveness as well as tumor growth in an autocrine fashion [152]. Furthermore, colon cancer cells express higher levels of endothelin receptors than normal colon epithelium [153], and plasma endothelin-1 levels are increased in patients with colorectal cancer [154], suggesting a similar autocrine function in this type of cancer, too.

Besides the aforementioned promigratory effects of chemokines and neurotransmitters on the migration of tumor cells, we have already mentioned that the opioid receptor MOR has an inhibitory effect on migration. We have delivered proof for this by the use of the specific MOR agonist DAMGO [120]. Furthermore, we have identified two neurotransmitters with an inhibitory function on tumor cell migration. The signaling of these neurotransmitters alone is not inhibitory, but it inhibits the effects of the above discussed stimulatory substances. The central nervous neurotransmitter gamma-aminobutyric acid (GABA) inhibits the norepinephrine-induced migration *via* the GABA-B receptor in tumor cells [155]. Baclofen is a GABA-B receptor agonist, which is approved for the treatment of spasticity [156]. This agonist inhibits the norepinephrine-induced migration of colon carcinoma cells *in vitro* [155], and reduces gastric and colon carcinogenesis when systemically administered in rats [157, 158]. Thus, GABA agonists might be useful pharmacological agents in the treatment of cancer, as has been discussed by Ortega [159]. We have observed a similar inhibitory effect on the norepinephrine-induced colon carcinoma migration by anandamide, which is the endogenous ligand for cannabinoid receptors [160]. Furthermore, cannabinoids inhibit the neoangiogenesis of malignant gliomas in mice [161]. Thus, in contrast to the chemokine system, where only

promigratory effects on tumor cells have been observed until now, the neurotransmitter system provides a matrix of positive and negative regulation of tumor cell migration.

Folkman *et al.* coined the term 'tumor-angiogenesis' over thirty years ago to describe the development of new blood vessels for the nourishment of tumor tissue [162]. These angiogenic factors are provided by the tumor cells themselves. Nerves likewise innervate tumors, and neurotrophic factors are secreted by tumor cells [163, 164]. Therefore, in analogy to the neoangiogenesis, we hypothesize that tumor cells initiate and nurture the ingrowth of nerve cells into the primary tumor, a process we hereby term 'neoneurogenesis'. These nerve cells produce neurotransmitters, which, as we have described above, might represent the initiation for metastasis development. We show in Fig. (1) that tumors of several tissues are susceptible for these neurotransmitters. Anti-angiogenic drugs are now under intense focus as therapeutic agents for cancer. We can similarly foresee the noneurogenesis as an active target for the inhibition of metastasis development.

SIGNAL TRANSDUCTION MOLECULES AS SPECIFIC TARGETS FOR THE INHIBITION OF TUMOR CELL MIGRATION

We have already discussed in the introduction section that the self-sufficiency of tumor cells in growth signaling can be inhibited by specific intracellular tyrosine kinase inhibitors. Similarly, a look at the signal transduction of tumor cell migration might provide access points to the specific inhibition of tumor cell migration. In doing so, it is essential that other migrating cells are not affected, especially those of the immune system. On the one hand, an advantage for the research in this field is that only a few specialized cells autonomously migrate in an adult organism, i.e. the tumor cells on the pathological side and the leukocytes, fibroblasts, sperm cells and stem cells on the physiological side [165]. On the other hand, the greatest disadvantage is that the intracellular mechanisms to generate locomotory force are almost the same among these cells [59]. Thus, we have to find molecules, which are specifically involved in the regulation of tumor cells.

We have recently provided a comparative review on the signal transduction of tumor cells and leukocytes [59], and have more elaborately discussed the signaling in tumor cells elsewhere [166]. To briefly summarize the signaling: Chemokines and neurotransmitters bind to receptors of the seven-helices family. The engagement of these receptors leads to the activation of heterotrimeric G proteins, which in turn activate two major pathways. These pathways lead to the activation of the protein kinase A and C (PKA and PKC), and an increase of cytosolic calcium. The activation of the PKC alone is sufficient to induce migration and consequently migration in leukocytes [167] and tumor cells [168], can be provoked by pharmacological activators such as phorbol esters. Interestingly, there seem to be differences in the PKC isotypes, which are engaged in these two cells types. The family of PKCs can be subdivided into classical, novel and atypical PKC isotypes, depending on their regulation by phospholipids and calcium [169]. In colon carcinoma cells, the classical PKC is the responsible signal

mediator for migration [168], whereas in T lymphocytes, a non-classical PKC isotype is active [170], as was shown by isotype-specific inhibitors in either case. Furthermore, T lymphocytes use integrin-independent strategies for migration, whereas for tumor cells these receptors are essential in order to facilitate the contact of the migrating cells to the environment, especially the extracellular matrix [171, 172]. In this context, it is noteworthy that an inhibitor for the expression of integrins [173], as well as a peptide inhibiting 1-integrin-mediated adhesion [174], are applied as patents.

There are probably more differences between tumor cells and leukocytes, which might provide future access to a selective inhibition of tumor cell migration. However, various aspects of the molecular regulation of cell migration are still unknown or under investigation. For example, downstream in the signal transduction, the activity of PKA and PKC in concert with the cytosolic calcium increase are translated into the generation of locomotory force by the activation of myosin as well as actin turnover and rearrangement [166]. The migration of leukocytes essentially depends on the activity of myosin II, whereas tumor cell migration utilizes myosin II-independent mechanisms, too [175]. The character of this myosin II-independent migration remains to be elucidated.

CURRENT AND FUTURE DEVELOPMENTS

We have discussed herein that stress and inflammation are two processes which have a strong impact on the development and progression of cancer. These two processes can be regarded as responses to influences from the environment on a psychological (stress) or immunological (inflammation) level. The molecular translation of these influences by neurotransmitters and hormones or chemokines and cytokines regulates several functions of cancer cells. The knowledge of this interconnection has already in part found entry into the clinical treatment of cancer, as we have discussed for the role of the cytokines EGF and IGF-I and -II. We herein enlighten the rationale for the use of clinically established seven-helices receptor blockers (e.g. beta-blockers), and for the development of new receptor blockers - as currently takes place especially in the chemokine system - for the inhibition of tumor cell migration. Since such substances are very interesting for the treatment of other types of diseases, too (e.g. psychological, neurological or immunological/infectious diseases), a broad interdisciplinary monitoring of pharmacological developments of seven helices receptor blockers or pharmaceuticals to associated molecules might lead to a mutual superimposed enrichment in all of these fields. As reviewed herein, the pharmaceuticals which are currently applied for patents with direct regard to the inhibition of metastases are mostly substances which aim at the proliferation or angiogenesis. The promising substances, antagonists to seven-helices receptors (chemokine and neurotransmitter receptors), might therefore constitute the first true class of metastasis inhibitors specifically targeting the migratory activity of tumor cells, and may potentially offer revolutionary approaches in oncological therapy.

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