MORPHINE-INDUCED ALTERATIONS OF TUMOR CELL ACTIVITY, AND TUMOR CELL-ENDOTHELIAL CELL INTERACTIONS: THE ROLE OF NITRIC OXIDE

by

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ABSTRACT

Morphine-induced alterations of tumor cell activity and tumor cell-endothelial cell interactions: the role of nitric oxide

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We examine the effects of morphine on THP-1 human monocytic leukemia cells, on B16F10 murine melanoma cells, and on the vascular endothelium of mouse aortic rings. Morphine had profound effects on both tumor cell lines. Morphine reduced cell proliferation and survival in both cell lines, and cell death was not mediated by apoptosis. Invasion of the TC across a fibronectin barrier was inhibited by morphine in both cell lines. This inhibition was only partially prevented by pre-treatment of THP-1 with either naloxone, or L-NAME. However, the inhibition of B16F10 cell invasion was completely abrogated by pre-treatment of these cells with naloxone, but unaffected by L-NAME. Thus, in THP-1 cells, morphine affected invasion

by both a NO-dependent and NO-independent mechanism; while in melanoma cells, morphine's effect appeared to be mediated solely by a NOS-independent, but opioid receptor-dependent mechanism. Morphine affected cell shape and actin polymerization in the THP-1 cell line, but had little or no effect on the B16F10. Morphine affected adhesion of B16F10 cells to aortic rings, and this effect was dependent on time after morphine treatment. Two hours after pre-treatment of aortic rings with morphine, adhesion was significantly increased when compared with saline treated aorta. 48 hours after pre-treatment of rings with morphine, adhesion was significantly decreased in both control and drug treated groups, but significantly fewer cells were seen in the morphine treated group than in the controls. The altered adhesion seen at both 2 and 48 hours was μ_3 and EC-NOS dependent. Experiments with RGD peptide suggest that B16F10 adhesion to the aortic endothelium is mediated by melanoma cell integrins, and that morphine treatment of the vascular endothelium impacts subsequent adhesion of endothelial cells to melanoma cell integrins. In summary, the results of this thesis demonstrate that morphine can directly affect tumor cell proliferation and viability, invasion, and tumor-endothelial cell adhesion. Our studies provide new and important information by demonstrating that morphine may act directly on tumor cells in either a NO-dependent or NO-independent manner, and on endothelial cells in a NO dependent manner modulating

properties of these cells that are capable of profoundly altering metastatic potential.

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INTRODUCTION

I. Overview

Morphine is widely used as an analgesic following a variety of surgical procedures and as a painkiller for cancer patients (Stevens *et al.*, 2000; Wagemans *et al.*, 1997). Several studies with morphine have indicated that this drug has the potential of either increasing or decreasing metastasis. While surgical stress has been shown to impair NK cell activity (Massi *et al.*, 2001; Page *et al.*, 2001) resulting in an increase in metastasis of a rat mammary adenocarcinoma (Page *et al.*, 1993), acute administration of morphine attenuated the surgically induced depression of NK activity and the concomitant increase in metastasis. However, other studies suggest that long term abuse of morphine can mediate immunosuppression by decreasing; CD4 and CD8 T cell numbers, as well as NK cell and macrophage activity (Novick *et al.*, 1989; Rahim *et al.*, 2001; Coussons-Read *et al.*, 2001; Hecks *et al.*, 2001; Bilfinger *et al.*, 1996; Tsai *et al.*, 2000). Thus, the effect of morphine on tumor cell (TC) metastasis is still ambiguous and needs to be evaluated.

Many biological effects of NO have been well documented. For example, NO modulates host immunity by regulating activities of macrophages, neutrophils and other immunocytes in migration, chemotaxis, proliferation

and differentiation, (Armstrong 2001; Coleman 2001; Park *et al.*, 2000; Abu-Soud *et al.*, 2000; Muhl *et al.*, 2000; Magazine *et al.*, 2000; Welters *et al.*, 2000; Opdahl *et al.*, 2000; Pio *et al.*, 1998; Beauvais *et al.*, 1995). NO has also been reported to induce angiogenesis by up-regulating $\alpha_v\beta_3$ integrin expression on endothelial cells and to promote migration and mitosis of endothelial cells (Lee *et al.*, 2000; Goligorsky *et al.*, 1999, Garcia-Cardena.and Folkman, 1998).

Increasing evidence suggests that NO has pleiotropic effects on diverse aspects of tumor biology. Although NO has been found to promote tumor development, its cytotoxicity on tumors and its anti-angiogenic activity have also been reported. (Xie *et al.*, 1998; Carretero *et al.*, 2001; Wang *et al.*, 2000; Powell, *et al.*, 2000). Overproduction of endogenous NO is autocytotoxic and can induce apoptosis and suppress tumor growth and metastasis, whereas low production of NO may protect tumor cells from apoptosis and promote tumor growth and development. It is possible that the effects of NO may be determined by the quantity of NO and the cell type exposed to it (Kim *et al.*, 2001).

Recently, Dr. Stefano characterized μ_3 opioid receptors expressed in human monocytes and in endothelial cells, and found that stimulation of this receptor by morphine was associated with NO production. (Stefano and Scharrer, 1996). As NO can affect tumor development and malignancy, we

speculate that morphine may modulate metastasis. Thus, the direct effects of morphine-induced NO release via μ_3 activation on metastasis needs to be explored and evaluated. With some cells, morphine-induced NO is able to induce cell conformational changes from amoeboid (for monocytes) or spindle shaped (for endothelial cells; EC) to round (Magazine *et al.*, 1996). Cell conformational changes are associated with cellular activities including migration, chemotaxis, phagocytosis, adhesion, and proliferation. Thus we propose that in modulating endothelial cell shape from spindle shaped to round, morphine may affect vascular-TC interactions. By inducing NO-mediated changes in the TC or EC morphine may facilitate TC adhesion to the endothelium and extravasation of the TC across the vessel wall, thereby promoting metastasis. In contrast, morphine may change one or more properties of the TC and/or EC and prevent TC adhesion to the endothelium thereby resulting in decreased extravasation and metastasis.

II. Role of morphine in the modulation of metastasis

Morphine is commonly utilized for postoperative pain management, and the antinociceptive properties of morphine have been suggested to provide therapeutic benefit by reducing the hormonal stress response to surgery (Page *et al.*, 1993; Bilfinger *et al.*, 1994; Fricchino *et al.*, 1994). Adequate pain intervention has been clearly associated with the decrease of a surgically induced immune suppression. Importantly, the failure to relieve pain following surgery results in a marked increase in pulmonary tumor cell retention in animal models (Bar-Yosef *et al.*, 1994; Page *et al.*, 2001). A reduction in NK activity observed following surgical stress has been demonstrated to result in a marked increase in metastatic development *in vivo.* (Pollock *et al.*, 1987; Yoshihara *et al.*, 1986; Ben-Eliyahu *et al.*, 1999; Zoller *et al.*, 1989). Since tumor metastatic potential can be increased by immunosupression, particularly suppression of NK activity, the restoration of NK activity associated with morphine analgesia would be expected to block surgery-induced suppression of NK activity, resulting in reduced tumor metastasis. Indeed, the metastatic potential the of the mammary adenocarcinoma tumor cell line, MADB106, a cell line sensitive to NK killing *in vivo* (Page *et al.*, 1993) is augmented following surgery whereas the increased pulmonary tumor cell retention observed 5 - 24 hours following surgery is abrogated by administration of morphine. This research

demonstrates that analgesic doses of morphine can attenuate the enhanced metastatic growth observed following surgery. Using B16F10 melanoma cells, Dr. Szalay has shown that a single analgesic dose of morphine administered prior to removal of ocular tumors from mice decreases spontaneous metastasis to the lung (Adjodha *et al.*, 2001; Szalay *et al.*, 2000; Adjodha *et al.*, MS in preparation). Thus, administration of morphine in adequate dose and timing may have potential therapeutic benefit for cancer patients.

III. Nitric oxide and metastasis

NO is a short–lived free radical which serves as a messenger molecule for diverse physiological and/or patho-physiological functions (Nathan *et al.*, 1994; Schmidt *et al.*, 1994). NO release can be produced by activation of one of the three known enzyme isoforms of nitric oxide synthase (NOS): NOSI (nNOS) and NOSIII (ecNOS) are constitutive isoforms that induce the production of NO for only short time periods (5-15 min.). NOSII (iNOS), an inducible isoform, must be up-regulated by agents such as cytokines, and catalyzes the release of NO for extended periods of time (24-72 hrs) (Nathon *et al.*, 1994). NOSI is expressed primarily in neuronal cells whereas NOSII and NOS III are expressed in a wide variety of cells (Table 1A).

NO is capable of increasing vasodilatation, capillary leakage, pulmonary edema, cell lysis, apoptosis, and of inhibiting platelet aggregation (Lowenstein *et al.*, 1994)(Schmidt *et al.* 1994) (Xie *et al.*, 1995) (Kubes *et al.*, 1994) (Kurose *et al.*, 1993). Furthermore, NO can be a potent inhibitor of cell spreading and attachment to the vascular endothelium and subendothelial matrix (Magazine *et al.*, 1996) (Stefano *et al.*, 1995). It has been reported that NO stimulates ADP ribosylation of actin and nitrosylation of various targeted substrates, resulting in inhibiting polymerization of actin filaments, cell adhesion, and migration (Stefano *et al.*, 1995; Clancy *et al.*, 1995; Burgstahler *et al.*, 1995; Marczin *et al.*, 1996). Exogenous and endogenous

NO released by leukocytes such as activated natural killer cells and macrophages can be cytotoxic to foreign intruders and TC. Furthermore, NO generated from the activated endothelium may also facilitate eradication of tumor cells in hepatic sinusoids (Wang *et al.*, 2000). Studies in mice have shown that increased expression of NOS inhibits tumor growth and metastasis (Xie *et al.*, 1995; Juang *et al.*, 1997; Xie and Fidler 1998)

NO has also been shown to promote TC growth and metastasis (Joshi *et al.*, 1997; Deliconstantinos *et al.*, 1994). It has been demonstrated that NO production is increased during metastasis and in cancer patients (Aris-Diaz *et al.*, 1994). A positive correlation of NOS expression with tumor progression has been reported in numerous human tumor models: tumors of the central nervous system (Cobbs *et al.*, 1995) gynaecological cancer (Thomsem *et al.*, 1994), breast cancer (Thomsem *et al.*, 1995), head and neck cancer (Gallo *et al.*, 1998) and prostate and bladder cancer (Klotz et al., 1999 and 1998). NO is responsible for vasodilatation, and may be able to trigger capillary endothelial cell growth and differentiation via endothelial-constitutive NO synthase activation, cyclic GMP (cGMP) elevation, mitogen activated kinase (MAPK) activation and fibroblast growth factor-2 (FGF-2) expression. (Ziche *et al.*, 2000) Therefore, NO may significantly contribute to angiogenesis. NO production from NO-producing TC induces the formation of new blood vessels around tumors. NO also promotes tumor blood flow via

dilation of arteriolar vessels and decreases leukocyte-endothelial adhesive interactions, ultimately leading to tumor growth by ensuring adequate nutrient supply through the tumor vasculature. Furthermore, high level NO production from TC may induce injury of endothelial cells thereby promoting TC extravasation.

Endogenous NO may play an important role in protecting TC from apoptosis induced by pro-apoptotic stimuli such as retinoid and cisplatin. (Melino *et al.*, 2000). The anti-apoptotic mechanism of NO may involve several pathways including inhibition of apoptotic signaling by the NO/cGMP pathway, inhibition of caspase activity by S-nitrosylation and regulation of antiapoptosis-related gene expression by NO. (Chung *et al.*, 2001). NO also acts as a scavenger of radicals to suppress the superoxide/hydrogen peroxide-mediated cytotoxicity towards TC. (Brune *et al.*, 1999 and 1997; Wink *et al.*, 1993). The anti-apoptotic and anti-necrotic effects of NO that allow TC to prolong survival rate in the circulation along with the property of promoting angiogenesis can contribute to advanced cancer development and metastasis.

IV. The inhibitory effect of morphine on tumor cell adhesion to the vascular endothelium

Although surgical stress has been associated with decreased immune function, the neuroimmune mechanisms that account for impaired inflammatory activity have yet to be rigorously evaluated. Receptors for the opiate alkaloid morphine have been intensively studied on neuronal cells. Morphine and opioid peptides including endogenous peptides with morphine-like activity bind to at least 3 main types of opioid receptors termed, μ, δ and κ which are unevenly distributed among various tissues of the body and exert a wide variety of biological effects including analgesia (Loh *et al.*, 1976) hypertension and hypothermia (Bloom *et al*, 1976) and immunomodulation (Jankovic *et al.*, 1994). However, recent studies have demonstrated the presence of an opiate alkaloid specific receptor that does not recognize opioid peptides, termed the μ_3 receptor (Stefano *et al.*, 1995, Makman *et al.*, 1995). It has been suggested that morphine induces NO release through μ_3 activation in various types of cells. Measurement of morphine-induced NO release in real time demonstrates that morphine stimulation of the μ_3 receptor is capable of eliciting the production of NO from a variety of cell types including rat, mouse and human endothelial cells (Fecho *et al.*, 1994; Stefno *et al.*, 1995; Magazine *et al.*, 1996), monocytes (Stefano *et al.*, 1995) and leukemia cell lines (Magrinat *et al.*, 1992) whereas activation of the classical μ, δ, and κ

receptors fails to induce NO release (Stefano *et al.*, 1995). Importantly the production of NO induced by morphine triggers marked changes in cell conformation within a variable period of time ranging from 20 minutes to 24 hours resulting in reduced cell spreading and impaired cell adhesion to matrix proteins and vascular preparations (Magazine *et al.*, 1996). In this regard several investigators have demonstrated that treatment of blood monocytes, leukocytes or leukemia cell lines with morphine markedly attenuates adhesion of these cells to human the saphenous vein and internal mammary artery (Magazine *et al.*, 1996; Ni *et al.*, 2000). Thus, it is possible that morphine may decrease TC adhesion to the capillary wall and provide therapeutic benefit in patients via induction of NO release following μ_3-receptor activation.

V. The significance of adhesion molecules, extracellular matrix proteins and integrins in tumor cell adhesion

For normal cells, growth and development are precisely controlled. However, malfunction of this mechanism can promote unlimited cell growth instead of differentiation, resulting in tumor formation. Tumors are categorized into two types: benign and malign. Benign tumors are able to divide locally, but cannot metastasize. Malignant tumors, however, are a major cause of death in humans, and malignant TC are capable of metastasis. During this process TC may lose contact with neighboring cells, migrate through the connective tissue, attach to and penetrate into blood vessels (intravasation), circulate throughout the body, leave the circulation (extravasation), induce angiogenesis, and colonize a secondary site. To a large extent, the cellular processes associated with metastasis depend on cytoskeletal remodeling. In fact, several cytoskeletal structural and regulatory proteins, such as E-cadherin, α catenin, β catenin, rac, rho, and integrins have been implicated in the metastatic process (Fukata *et al.*, 2001; Loric *et al.*, 2001; Tomlinson *et al.*, 2001; Weed *et al.*, 2001; Del Peso *et al.*, 1997). During extravasation, the interaction of TC with the vascular endothelium of a target organ is thought to be a crucial event. It has been reported that various types of integrins, adhesion molecules such as P- and E- selectin, ICAM-1, ICAM-3, VCAM, and ECM proteins such as fibronectin and

vitronectin are associated with and/or required for TC adhesion and invasion. (Alexiou *et al.*, 2001; Ding *et al.*, 2001; Qian *et al.*, 2001; Uotani *et al.*, 2001; Borsig *et al.*, 2001; Seftor *et al.*, 1999; Dommann *et al.*, 1997; Tawil, *et al.*, 1996) Furthermore, it has been suggested that matrix metalloprotinases (MMP) have to be secreted to enzymatically digest ECM proteins during TC invasion (Bodey *et al.*, 2001; Chang *et al.*, 2001).

Integrins are a class of membrane-spanning glycoproteins that typically link the cytoskeleton to the extracellular environment, but may also play a role in cell-cell adhesion (Hynes *et al.*, 1987). These molecules, consisting of a heterodimer of noncovalently linked α and β subunits, bind to a variety of extracellular ligands including extracellular matrix proteins, complement components and cell surface adhesive molecules (Table 1B and 1C). Integrins include the fibronectin receptor and related receptors that bind to collagen and laminin, a receptor on platelets that binds fibronectin and fibrinogen, and receptors on leukocytes that play a role in the adhesion of neutrophils to endothelial cells. The integrins recognize a specific tri-peptide amino acids sequence Arg-Gly-Asp (knows as RGD) common to a number of extracellular adhesive proteins (Hayman *et al.*, 1985; Pierschbacher *et al.*, 1985). Several integrins associated with TC adhesion include $\alpha_2\beta_1$and $\alpha_3\beta_2$ in the prostate cancer cell line PC3 (Kiefer *et al.*, 2001; Coppolino *et al.*, 1999), $\alpha_6\beta$ in pancreatic cancer cell line, and $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ in

melanoma cells (Li *et al.*, 2001; Voura *et al.*, 2001; Elshaw *et al.*, 2001). Activation of integrins not only facilitates TC adhesion but also switches on several enzymes involved in signal transduction pathways, such as tyrosine kinases, ras and rho small GTPase, PI3 kinase as well as protein kinase C, resulting in regulation of gene expression and promotion of TC growth and migration (Gu *et al.*, 2001. Trusolino *et al.*, 2001; Berrier *et al.*, 2000).

The cadherin family of cellular adhesion receptors including in E, P, N, L subclass, is important for cell–cell interactions, especially in homophilic binding in which they bind selectively to identical cadherin types (Takeichi *et al*, 1991; Nose *et al.*, 1990). It has been demonstrated that the dissociation of cadherin interactions or changes in cadherin expression have a direct effect on cell morphology and may contribute to the release of tumor cells from the primary site and be associated with a more aggressive phenotype.

Members of the selectin family include L-selectin, P-selectin and E-selectin. Selectins are associated with adhesion and rolling of leukocytes along the inner vessel wall (Frenette *et al.* 1995; Norman *et al.*, 1995. Ley *et al.*, 1995; McEver *et al.*, 1995). Selectins are important in guiding circulating neutrophils towards areas of damaged tissue in an acute inflammatory process. Recently, it has been reported that P-selectin mediates adhesion in the human melanoma cell line NKI-4, and E selectin mediates breast cancer cell adhesion to the endothelium (Kaytes *et al.* 1998; Narita *et*

al., 1996). It also has been shown that over expression of P- and E-selectin occurs in micro-vessels near breast cancer cell tumors and suggests that selectin may promote angiogenesis by increasing adhesion and migration of endothelial cells. Therefore, selectin expression may alter TC adhesion and metastasis.

In general, attachment of circulating cancer cells to the vessel wall during extravasation is a prerequisite for the their entry to the extravascular space and is the one of the most important steps of metastasis. This process requires engagements of various extracellular adhesion molecules and their counterpart or cognate receptors. Therefore any means of decreasing the interaction of TC and endothelial cells may help prevent metastasis.

VI. Summary

The sections above illustrate the potential importance of morphine and NO on TC function, and on metastasis. In the present thesis, I evaluate the direct *in vitro* effects of morphine on NO production, and on the conformation, proliferation, viability, and invasion of B16F10 melanoma and the THP-1 leukemic cell lines. In addition, I evaluate the effect of morphine and NO on the adhesion of melanoma cells to the endothelium of the mouse aorta.

Table 1A. Isoforms of Nitric Oxide Synthase (NOS)

Name	Previous Name	Type/Mr (kda)	Regulation	Presence	Human chromosome	Duration
NOS1	Neuronal NOS nNOS	Constitutive 130	Ca^{2+} /calmodulin	Brain, cerebellum other neuronal tissues	12q24, 1-12q24.31	< 1 hour
NOS2	Endothelium NOS eNOS	Constitutive 135	Ca^{2+} /calmodulin	Endothelial cells	7q35-7-7q36	< 1 Hour
NOS3	Inducible NOS iNOS	Inducible 160	Endotoxin cytokines	Macrophages Neutrophils Chondrocytes Hepatocytes	17q11.2	>> Hours

Table 1B. Integrin family of adhesion receptors

Dimers	Ligands	Distribution
$\alpha_1\beta_1$	Laminins(E1), Collagen I, IV.	Small vessel endothelium, smooth muscle, fibroblasts.
$\alpha_2\beta_1$	Laminins(E8), Collagen	Epithelia, platelets, fibroblasts.
$\alpha_3\beta_1$	Laminins(E8), Nidogen, Fibronectin (Arg-Gly-Asp), Collagen, Invasin.	Smooth muscle, connective tissue, keratinocytes, kidney.
$\alpha_4\beta_1$	Fibronectin (Leu-Asp-Val), VACM-1.	Leukocytes, developing skeletal muscle.
$\alpha_5\beta_1$	Fibronectin (Arg-Gly-Asp), L1, Invasin.	Widespread.
$\alpha_6\beta_1$	Laminins(E8), Frtilin, Invasin.	Epithelia, vessel wall, nerves and oocytes.
$\alpha_7\beta_1$	Laminins(E8).	Skeletal muscle, vessel wall.
$\alpha_8\beta_1$	Tenascin..	Nervous tissue, Epithelia and muscle.
$\alpha_9\beta_1$	Tenascin.	Epithelia and muscle.
$\alpha_L\beta_2$	ICAM-1, ICAM-2, ICAM-3.	Lymphocytes, myeloid cells.
$\alpha_M\beta_2$	C3bi, Fibrinogen, Heparin, Haptoglobin, Hemagglutinin.	Myeloid cells, lymphocyte subsets.
$\alpha_X\beta_2$	C3bi, Fibrinogen..	Myeloid cells, lymphocyte subsets.
$\alpha_D\beta_2$	ICAM-1, ICAM-3.	Tissue macrophage.
$\alpha_E\beta_7$	E- Cadherin.	Lymphocytes, macrophage.
$\alpha_4\beta_7$	Fibronectin(Leu-Asp-Val), VCAM.	Lymphocytes.
$\alpha_v\beta_1$	Fibronectin, vitronectin(Arg-Gly-Asp).	Fibroblasts, neuroblastoma, 293 kidney cells *in vitro*.
$\alpha_v\beta_3$	Vitronectin(Arg-Gly-Asp), Fibrinogen, von Willebrand, Fibronectin(Leu-Asp-Val), Denatured collagen and Laminin, Osteopontin, Tenascin, Thrombospondin, PECAM-1.	Endothelium, osteoclasts, connective tissue.
$\alpha_v\beta_5$	Vitronectin.	Epithelia.
$\alpha_v\beta_6$	Fibronectin, Tenascin.	Epithelia.
$\alpha_{IIb}\beta_3$	Fibronectin, von Willebrand Vitronectin, Fibronectin(Leu-Asp-Val).	Platelets, and melanoma cells.

Table 1C. The integrins expressed on B16F10 or THP-1.

Integrin	B16F10	THP-1
$\alpha_4\beta_1$	x	x
$\alpha_5\beta_1$		x
$\alpha_M\beta_2$	x	
$\alpha_X\beta_2$	x	
$\alpha_E\beta_7$	x	
$\alpha_4\beta_7$	x	
$\alpha_v\beta_3$	x	x
$\alpha_{IIb}\beta_3$	x	x

CHAPTER ONE

NITRIC OXIDE PRODUCTION FROM THP-1 AND B16F10 CELLS FOLLOWING MORPHINE EXPOSURE

Introduction

Morphine is a naturally occurring substance in the opium poppy, *Papaver somniferum*. It exerts multiple biological effects on target tissues by interacting with cell surface receptors including μ–, δ– and κ– opioid receptors (Table 2.1)(Kieffer, *et al.*, 1994; Gaveriaux, *et al.*, 1995; Piros, *et al.*, 1996; Standife, *et al.*, 1997; Raynor, *et al.*, 1996). Morphine is a potent analgesic, and its primary clinical use is in the management of moderate to severe pain. Morphine is widely used for postoperative and intractable pain management, especially for patients with cancer. Nonetheless, the direct biological effects of morphine on tumor cells have not been well characterized.

Recently a novel μ subtype opioid receptor designed as a μ_3 has been discovered in human endothelial cells (Stefano, *et al.*, 1995), inflammatory cells and microglia (Magazine, *et al.*, 1996). This receptor is specific for

morphine and does not bind opioid peptides. It has been demonstrated that morphine binding to the μ_3 receptor is capable of stimulating of nitric oxide (NO) production.

NO, an endothelium-dependent relaxing factor (EDRF), was first discovered by Dr. Furchgott in the early 1980's (Furchgott, 1999). NO is a short-live, gaseous radical that is the smallest biosynthetically derived secretary product of mammalian cells and is an identified messenger molecule with diverse functions through the body. NO is released as a metabolic product from several types of cells including endothelial, epithelial, neuronal, and phagocytic cells. In blood vessels, NO contributes to the regulation of blood pressure by inducing vaso-relaxation (Nathan, 1992; Xie *et al.*, 1994). It also inhibits blood clot formation by inhibition of platelet aggregation (Moncada *et al.*, 1991; Moncada, 1992; Ignarro, *et al.*, 1989). In macrophages, NO mediates tumoricidal and bactericidal action (Nathan *et al.*, 1991). In the brain and peripheral nervous system, NO functions as a neurotransmitter, and is believed to be associated with stroke and neurodegenerative diseases (Hope *et al.*, 1991; Nowicki *et al.*, 1991; Meldrum *et al.*, 1900). In addition to these biological functions, NO regulates the following: activation of transcription factor (Kroncke, *et al.*, 2001); mutagenesis (Inoue, *et al.*, 1995); apoptosis (Dimmeler, *et al.*, 1997); glycolysis and mitochondria electron transport (Albina, *et al.*, 1993; Bolanos,

et al., 1994); fusion of myoblasts (Lee, *et al.*, 1997); the contraction of muscle in the stomach, intestine uterus and heart (Lefebvre, 1998; Kohjitani, *et al.*, 2001; Imbrogno, *et al.*, 2001); erection of the penis (Khan, *et al.*, 2000); opioid dependence, tolerance and toxicity (Herman, *et al.*, 1995); adhesion of blood platelets, neutrophils, and leukocytes (Loscalzo, *et al.*, 2001; Thom, *et al.*, 2001; Ramamurthi, *et al.* 1999; Lin, *et al.*, 2001; Lindemann, *et al.*, 2000), and proliferation of myeloid progenitors, T cells, and tumor cells (TC) (Schattne,r *et al*, 2001; Maciejewski, *et al.*, 199; Brennan, *et al.*, 2001), and alters metastasis and angiogenesis.(Ziche *et al.*, 2000; Fukumura *et al.*, 2001; Carretero, *et al.*, 2001; Lee *et al.*, 2000).

It has been reported that nitric oxide synthase (NOS), specifically iNOS (NOS2) is strongly expressed in invasive melanomas and other tumors (Massi, *et al.*, 2001), and is capable of regulating metastatic potential (Xi and Fidler, 1998). High level of NOS expression in numerous malignant tumors cells may be associated with decreased or increased metastatic potential (Ekmekcioglu, *et al.*, 2000). We postulate that morphine coupling with NO release from tumor cells through μ_3 activation might impact metastatic potential. We evaluate two cell lines, THP-1, a human leukemic cell line and B16F10 melanoma cell line. to determine TC capability of responding to morphine, and of producing NO in response to morphine.

Material and Methods

Animal.

C57BL/6 mice and C57BL/6.129P2-NOS3^{tm1Unc}, NOS3 knockout mice were purchased from Jackson Laboratories (Jacksonville, Florida). Mice were 8 – 10 weeks of age when used in experiments.

Cell cultures.

The murine B16F10 melanomas were obtained from Dr. Szalay of Queens College, CUNY. Cells were maintained in DMEM (Gibco, Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS). Cells were used of between passage 5 to 10. THP-1, a human monoblastic cell line, was obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640(sigma) supplemented with 10 % FBS, 0.1mg/ml streptomycin, 100 U/ml penicillin G and maintained at 5 % CO_2 / 37°C. (Life Technologies, Grand Island, NY)

Nitric Oxide (NO) Measurements in cell culture.

THP-1 and B16F10 cells were harvested using trypsin, 0.25% and washed extensively with PSS just before used in assays. Approximately 10^6 THP-1 or B16F10 cells were placed in 5 ml of aerated (95%O_2/5%CO_2) PSS buffer (130 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 14.9 mM $NaHCO_3$, 5.5 mM Dextrose, 0.03mM EDTA, and 2.5 mM $CaCl_2$), pH 7.4 and maintained at 24°C, followed by evaluation of NO release using a N-

selective amperometric probe, as descried Previously (Magazine *et al.*, 1995, 1996).

The detection of NO release from C57Bl/6 or NOS3 knockout mice, C57BL/6.129P2-NOS3^{tm1Unc}

Normal and transgenic NOS knockout mice were sacrificed by intraperitoneal injection with 0.25 ml pentobarbital sodium (120mg/kg) followed by rapid excision of the thoracic aorta. The vessels were placed in ice-cold PSS, pH 7.4 and excess connective and adipose tissue was removed. The aorta was cut into 2- to 3-mm segments and placed in 5 ml of aerated (95%O_2-5%CO_2) PSS maintained at 24°C for evaluation of NO release. Aortic segments denuded of endothelium were prepared by insertion of a smooth metal wire into the lumen of the vessel followed by gentle scraping of the tissue (King et al., 1997). Stimulation with 10 nM Acetylcholine (ACh) was employed to confirm functional denudation of the tissue (Magazine, 1995).

Results

Morphine induced NO release from THP-1 cells. Stimulation with 1 μM morphine induced NO release that was detected 2 to 3 minutes following stimulation and maximal peak release of NO was approximate 35 nM in 5 minutes (Figure1.1A). NO release induced by morphine was abrogated in the presence of the specific NOS inhibitor, N^{w}-nitro-L-arginine methyl ester (L-NAME, Fig. 1.1B). In contrast, little or no NO production from B16F10 melanoma cells stimulated by morphine was detected (Figure 1.2).

Discussion

Morphine's capacity to stimulate increased production of NO was evaluated in a leukemic and melanoma cell line. We demonstrated that morphine induced detectable levels of NO release from THP-1 but not from B16F10 cells. The morphine-induced NO release from THP-1 cells was abrogated by pretreatment with the NOS inhibitor, L-NAME, demonstrating the specificity of the response. Previous studies have demonstrated that human monocytes express μ_3 opioid receptors on their cell surface and this receptor is associated with NO production following morphine engagement. THP-1 is the acute monocytic leukemia cell line derived from a boy at age of 11. Our results suggest that the μ_3 opioid receptor is likely also expressed on the THP-1 cell surface. The duration of NO production catalyzed by NOS1 and NOS3 is about 10 to 20 minutes form, while NO release from NOS2 lasts for several hours. The results demonstrate (Figure 1.1A) that the duration of NO release is quite short, about 10 minutes, suggesting NO production by constitutive nitric oxide synthase activation. Although the duration of NO release is short, such release is sufficient to induce physiological change in THP-1 as well as in monocytes (Magazine *et al.*, 1996).

In contrast, morphine stimulation of NO production from B16F10 was not detected (Figure 2). The cell line may not release NO in response to morphine and may lack the μ_3 receptor. Alternatively, NO may be released, but may be

below the limit of detection by the NO sensor. The amperometric probe is not able to measure less than a 1-2 nM increase in NO levels (Magazine, 1995). Therefore, we cannot discount the possibility that morphine induces a low level NO release from B16F10 cells.

For μ_3-bearing TC lines, morphine may modulate cellular activity via NOS activation. Although, morphine-induced NO modulation of cellular activities is not likely in B16F10 TC, morphine may nonetheless modulate B16F10 cell activity via opiate receptors, instead of μ_3. It has been reported that morphine inhibits proliferation of human prostate, small lung cancer and non-small lung cancer as well as breast cancer cell lines (Kampa, *et al.*, 1997). However, these TC have not been examined for μ_3 expression. Taken together, we conclude that at least in the case of THP-1, morphine may modulate TC physiological activity in an NO-dependent way.

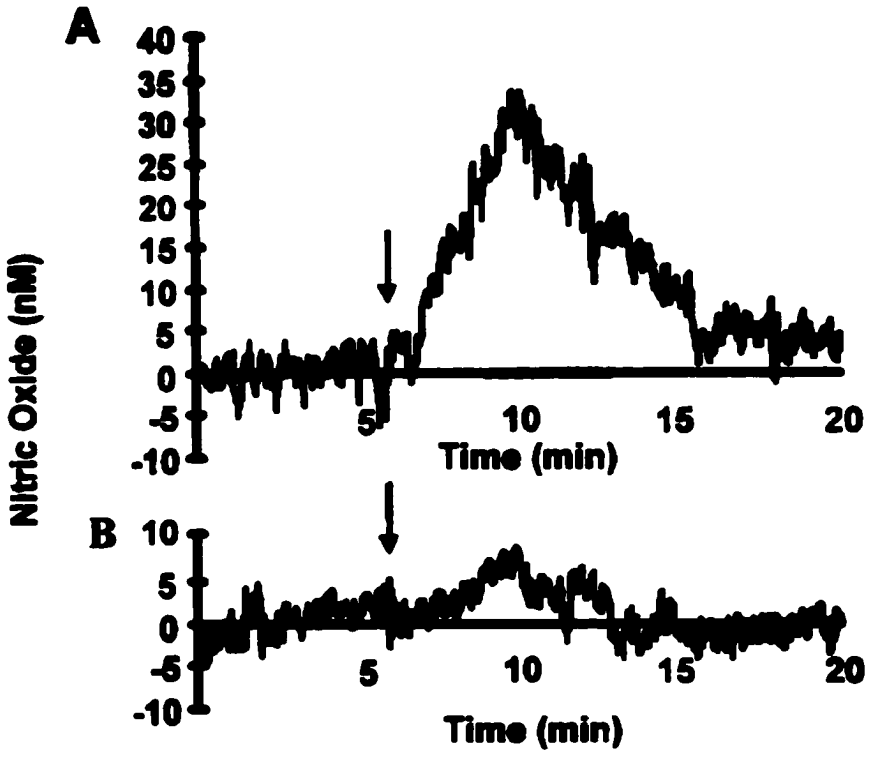


Figure 1.1A and 1.1B. Real-time analysis of morphine-induced NO release by THP-1.

NO measurement was carried out using NO –specific amperometric probe. Morphine induces nitric oxide release from THP-1 cells and NO release was lasting for 10 minutes and the maximal concentration of NO release from cells stimulated by 1 μM is roughly 35 nM. (Figure 1.1A). NO release from THP-1 was abrogated, when cells were pretreated with nitric synthase inhibitor, L-NAME following morphine exposure (Figure 1.1B).

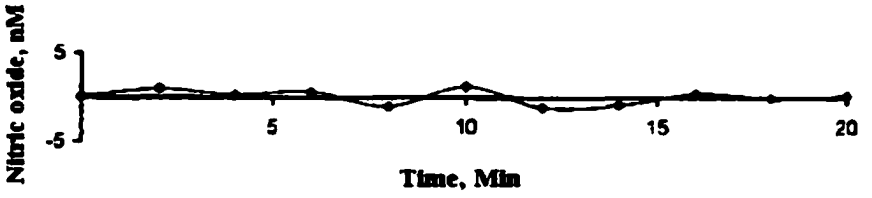


Figure 1.2. Measurement of nitric oxide (NO) release from B16F10 melanoma cells stimulated by morphine.

No significant nitric oxide release from B16F10 stimulated by 1 μM morphine was detected.

Table 2. Principle sites of endogenous and exogenous opioids and their antagonists at various classes of opioid receptors

Receptor	Agonist	Antagonist
μ	β-Endorphin	Naloxone
	Endomorphine	Naltrexone
	Morphine	Naloxonazine
	Pethidine	β-Funaltrexamine
	Methadone	Nalorphine
	Fentanyl	Pentazocine
	Ethorphine	
μ1	N-(2-pyrazinyl)-N-(1- phenethyl-4-piperidinyl)-2 -furamide	Naloxonazine
μ2	?	N-(2-pyrazinyl)-N-(1- phenethyl—4- piperidinyl)-2- -furamide
μ3	Morphine	Naloxone
κ	Dynorphin	Naloxone
	Morphine	Naltrexone
	Pentazocine	
	Nalorphine	
	Nalbuphine	
	Etorphine	
δ	Leu-enkephalin	Naloxone
	Met-enkephalin	Naltrexone
	DeltrophineII	
	Etrophine	

CHAPTER TWO

CELLULAR CONFORMATIONAL CHANGE OF THP-1 AND B16F10 FOLLOWING MORPHINE EXPOSURE

Introduction

Precise control of cellular conformation is essential for the proper functions for various cell types. Some cells respond to surfaces coated with ECM proteins by adhering and then spreading out to acquire a flattened morphology. Improper regulation of cellular morphology and cell to cell or cell to matrix connections has frequently been associated with cancer and other diseases. Cellular conformational change induced by exposure of inflammatory cells, vascular smooth muscle and neurons to diverse signal molecules or agonists including morphine has been reported (Stefano *et al.*, 1989, 1994 and 1995).

In chapter one, we have demonstrated that morphine-induces NO production from THP-1 cells. NO is a signal molecule that triggers the alteration of cell shape. Cell conformational change from amoeboid or spindle to round induced by NO has been reported in human monocytes,

granulocytes, endothelium and aortic smooth muscle cells (Magazine *et al.*, 1996; Hassid *et al.*, 1995). NO-induced cell shape changes are associated with the inhibition of cytoskeletal-associated actin polymerization. The mechanism of the NO-induced augmentation of F actin de-polymerization results from the ADP-ribosylation of monomeric actin and the prevention of actin filament formation. (Gorodeski, 2000; Forslund *et al.*, 2000; Jun *et al.*, 1996; Clancy *et al.*, 1995). The actin cytoskeleton is a major determinant of changes in cell shape, and interactions between actin filaments and the cell membrane are essential for cell adhesion, spreading and migration, as well as for signal transduction. (Hall *et al.*, 1998; Schoenwaelder and Burridge; 1999). The initiation of the apoptotic process in human epithelial cells by actin-derangement also has been reported (White *et al.*, 2001). This suggests that cyto-architecture may determine cell fate and activity. Thus, a morphine-induced cellular conformation change mediated by NO may regulate THP-1 adhesion, migration and proliferation. Although B16F10 melanoma cells may not be capable of morphine-induced NO production, morphine may induce cell shape change through other mechanism and affect cell activities. Thus, alterations of cell shape in B16F10 cells following morphine exposure also needs to be evaluated.

Material and Method

Evaluation of cell conformation.

THP-1 and B16F10 cells were washed extensively with DMEM and RPMI 1640 respectively, diluted to a concentration of 10^6 cells/ml and maintained at 4°C prior to use. Cell suspensions were grown on glass cover slips coated with 5-μg/ml fibronectin whereupon changes in cell conformation was followed for approximately 1 hour at 24°C as described previously, and the degree of conformational change is based on measurements of cellular area and perimeter by using image analysis software analysis (Image Analytics, Happauge NY) as descried previously (Magazine *et al.*, 1996) (Stefano *et al.*, 1995). Changes in cell conformation are evaluated by using phase-contrast microscopy with frame grabbing and computer analysis of changes in cellular conformation. Conformation ranged from inactivate-rounded to active-spindle shaped and were determined by measurements of cellular area and perimeter and mathematically expressed by use of the form-factor (FF) formula: $(4x\pi A)/P^2$, where A is the area of the cell and P is the perimeter. The lower of the FF value, the higher the cellular perimeter and the more spindle or amoeboid the cellular shape (Stefano *et al.*, 1995). The proportion of activated (spindle and amoeboid) cells was determined by noting the proportion of spindle or amoeboid to round cells using phase contrast microscopy.

Morphine-induced changes in cell conformation were evaluated as follows: After the addition of 1μM morphine to cells that had been allowed to adhere to fibronectin coated cover slips for 30 min, changes in cell conformation were evaluated for an additional 30 min. For experiments in the presence of opiate receptor antagonists or NOS inhibitors, the agent was added to the cell suspension just prior to addition of the cells to slides. Slides were then evaluated for changes in cell conformation. (Magazine *et al.*, 1997)

Statistical analysis

Results are expressed as the mean plus SE. Differences among groups were evaluated by using ANOVA test. $P < 0.05$ is considered to be statically significant.

Results

To evaluate the effect of morphine-induced cellular conformational changes in THP-1 cells, THP-1 cells were placed on the fibronectin-coated coverslip for 1 hour following morphine exposure, and then the cell shape was evaluated. Exposure of THP-1 to 1 μM morphine resulted in significant reduction in the number of amoeboid cells and adoption of rounded conformation (Figure 2.1 A) and the Form Factor ($FF= 4x\pi A/P^2$, where A is the area of the cell and P is the perimeter) increases to 0.78(Figure 2.1B). To evaluate the role of NO in morphine-induced cell conformational change, THP-1 cells were pretreated with L-NAME followed by addition of morphine to cells. The FF of THP-1 pre-treated with L-NAME dropped to about 0.25, which is near to that observed in the control (Figure 2.1B). These results suggest that morphine-induced rounding of THP-1 cells was abrogated by pretreatment with L-NAME and that NO mediated the cell shape change induced by morphine. Results with B16F10 melanoma cells show that the FF is not significantly changed following exposure to morphine (Figure 2.2B), and that morphine does not induce B16F10 cell shape changes (Figure 2.2A).

Discussion

The present study demonstrates that morphine induces THP-1 cell shape change from amoeboid shaped to round. The morphine-induced cell conformational change in THP-1 cells is significantly abrogated by L-NAME, a NOS inhibitor. Thus, the effect of morphine on cell conformation is suggested to be mediated by NO. Furthermore, the cell conformational change of THP-1 induced by morphine is also inhibited by the antagonist of opiate receptors, naloxone (Cohen, unpublished data). This suggests that morphine mediates NO release from THP-1 via opioid receptors. The observation that morphine-induced cell rounding and NO release from THP-1 is abrogated in the presence of L-NAME and naloxone is consistent with previous report in which morphine induced cell conformation changes in human monocytes were mediated by NO (Magazine *et al*, 1996).

However, morphine cannot induce B16F10 cell shape change. Concerning the sensitivity of B16F10 to morphine, B16F10 cells were treated with morphine various concentration from 0.1 to 10 μM and the results all remained the same, which, morphine cannot promote cell rounding on B16F10. In the previous chapter, we have demonstrated that morphine is not able to induce NO release from these cells. Thus, this data suggests that B16F10 melanoma cells lack NOS expression and/or μ_3 receptors.

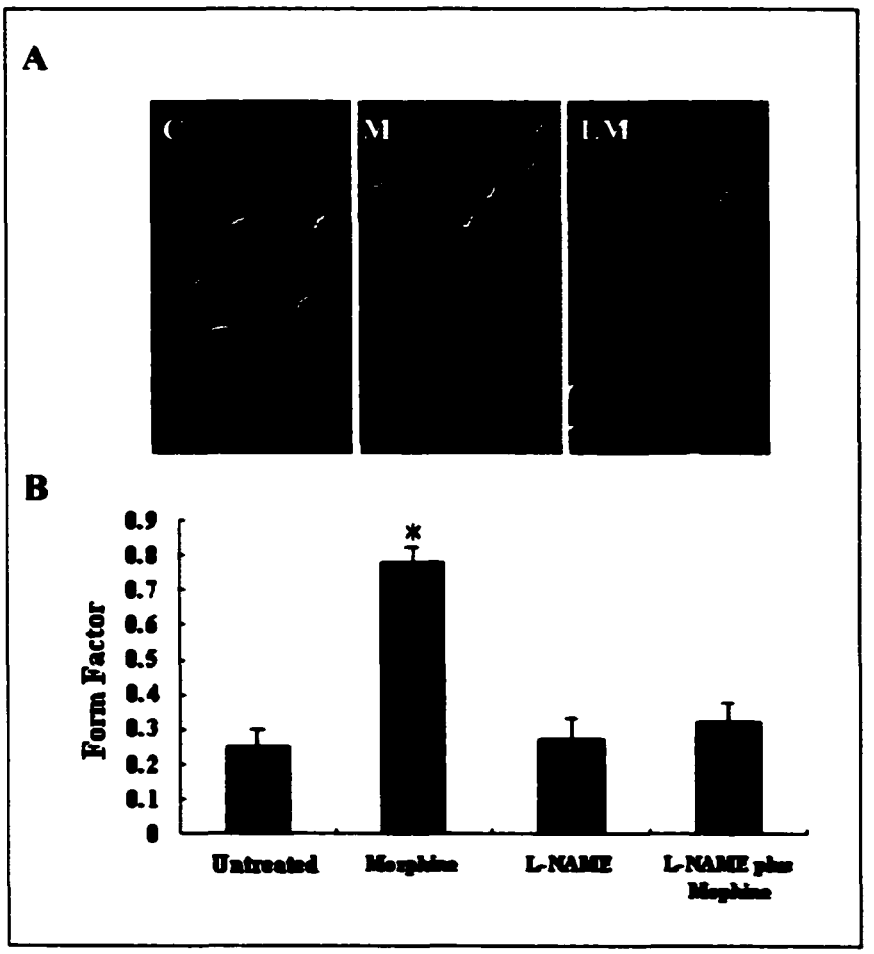


Figure 2.1A and 2.2B. Morphine-induced cellular conformational change in THP-1 cells.

Cellular conformation was evaluated by using phase-contrast microscopy with Image Analytics software (Hauppauge, NY). Change in cellular conformational, which ranged from inactivated-rounded to activated-spindle,

were determined by measurements of cellular area and perimeter and were mathematically expressed by the form –factor (FF) formula $(4x\pi A)/P^2$, where A is the area of the cell and P is the perimeter. The lower this number (FF), the high the cellular perimeter and the more amoeboid cellular shape. Morphine stimulates THP-1 cell shape from amoeboid to round. THP-1 cells were placed on the fibronectin-coated coverslip for 30-minute adherence followed by exposing in 1 μM morphine. Cell shape was evaluated 1 hour following morphine exposure. The result shows that morphine induces THP-1 cell shape changes from amoeboid to round (Fig. 2.1A-M) and increases FF roughly from 0.25 to 0.78(Fig.2.1B) and however, the morphine-induced cell rounding is abrogated by L-NAME (Fig. 2.1A-LM). The data in the figure are given as medians. The data resulted from 5 independent experiments. *Denotes statistical significance; *P<0.001. The Form Factor was analyzed for statistical significance by using ANOVA test.

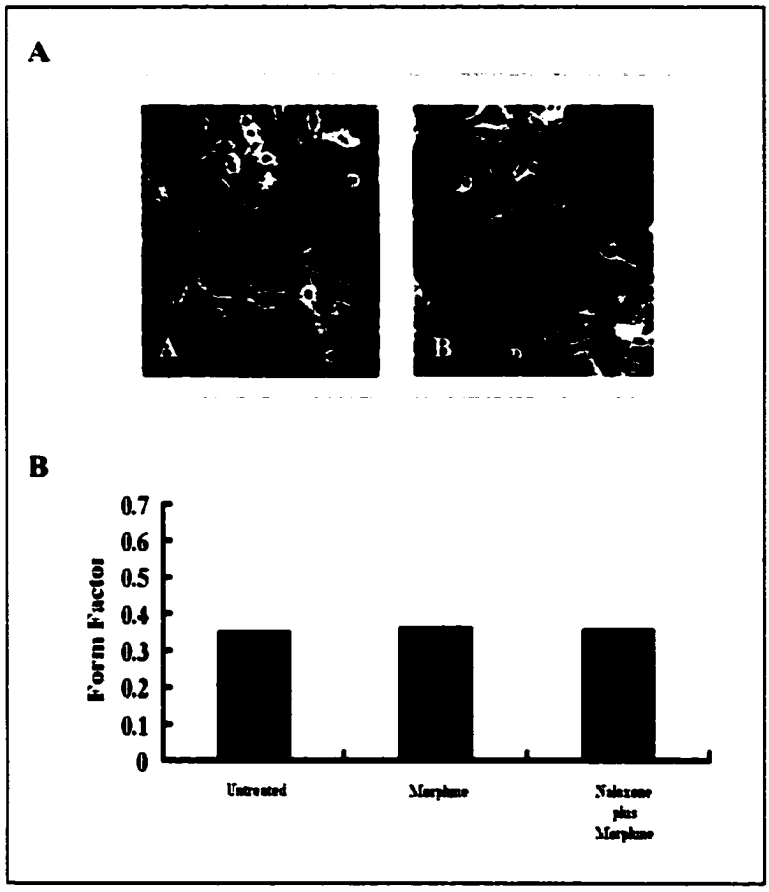


Figure 2.2A and 2.2B. Lack effect of morphine on cellular conformational change of B16F10 melanoma cells.

Morphine does not induce B16F10 cellular conformational change. B16F10 cells were placed the fibronectin-coated coverslip for 30-minute adherence followed by exposure to 1 μM morphine. Cell shape was evaluated 1 hour following morphine exposure. The result shows that morphine does

not induce B16F10 cell shape change (Fig. 2.2A, M); cells still remain spindle shaped and 0.35 FF after exposure to morphine (Fig. 2.2B). The data in the figure are given as medians and are the result of 7 independent experiments. The Form Factor was analyzed for statistical significance using the ANOVA test.

CHAPTER THREE

THE EFFECTS OF MORPHINE ON CELL PROLIFERATION, VIABILITY, ACTIN POLYMERIZATION AND MOTILITY IN THP-1 AND B16F10 CELLS

Introduction

In addition to its analgesic and immunosuppressive property, morphine has been demonstrated to act as an antitumor agent in *vitro* and *in vivo*. Morphine inhibits neoplasia growth by decreasing cell proliferation in a dose-dependent and reversible manner (Kampa *et al.*, 1997; Sergeeva *et al.*, 1993; Aylsworth *et al.*, 1979: Ilyinsky *et al.*, 1987: Kikuchi *et al.*, 1987; Kita *et al.*, 1992; Murgo, 1989; Scholar *et al.*, 1987). Furthermore, it has been suggested that induction of apoptosis and inhibition of NF-κB may contribute to the antitumor effect of morphine on several cell lines including HL-60, a promyelocytic leukemia cell line, U251, a glioblastoma cell line PC-9, a lung cancer cell (Sueoka *et al.*, 1998). This evidence is based on the discovery of two morphine-induced cachexia-inducing cytokines, TNF-α and leukemia inhibitory factor, and LIF from cancer cells, which are able to induce

apoptosis on TC (Sueoka *et al.*, 1996). Recently, the inhibition of growth induced by morphine also has been observed from various human cancer cell lines (Maneckjee *et al.*, 1990; Hatzoglou *et al.*, 1995). Furthermore, the observation that morphine suppresses tumor growth and metastasis also has been reported (Kuraishi *et al.*, 2001).

It has previously been shown that morphine induces cell conformational changes in various cells including human monocytes, granulocytes, endothelial cells, invertebrate immunocytes and microglia (Magazine *et al.*, 1996). In the present thesis, our observations suggest that morphine may induce cell shape change via NOS activation coupled with NO release. Morphine may potentially alter diverse cellular function via NO targeting of various intracellular proteins. Morphine may modify targeted proteins by virtue of the ability of NO to nitrosylate intracellular thios such as glutathione, and protein kinase C, or to react with Fe-containing enzymes such as guanylate cyclase and aconitatase (Clancy *et al.*, 1994; Gopalaskrishna *et al.*, 1993; Stamler *et al.*, 1992). An additional mechanism of modulating targeted proteins by NO is the ability of this molecule to induce ADP-ribosylation (Clancy *et al.*, 1993; Sheffler *et al.*, 1995; Brune *et al.*, 1989). NO inhibits actin filament polymerization by inducing ADP-ribosylation on G-actin resulting in inhibiting polymerization or cap formation (Lee *et al*, 1997; Clancy *et al.*, 1995). NO–induced F-actin

disassembly is mediated via cGMP, cAMP, and protein Kinase A activation. (Sandau *et al.*, 2001). Exogenous NO generated by SNAP is also able to induce depolymerization of cytoskeletal actin filaments and inhibit migration and chemotaxis of human peripheral blood monocytes (Magazine *et al.*, 2000). These observations suggest that endogenous and exogenous NO may modulate several cell activities via depolymerization of cytoskeletal actin filaments in cells.

Cell shape is maintained by a cytoskeletal network, including actin filaments, microtubules, and intermediate filaments and associated proteins. Alteration of the cytoskeleton induced by internal or external stimuli can modulate cell growth, proliferation, apoptosis, differentiation, motility, signal transduction, gene expression, chromosome movement and extracellular matrix remodeling (Chicurel *et al.*, 1998; Penman *et al.*, 1983; Birchmeier *et al.* 1984). Thus, cytoskeletal proteins play a pivotal role not only in the regulation of cell shape but in various other cellular activities as well.

In the present chapter we examine the ability of morphine to affect cell proliferation, viability, apoptosis, actin polymerization, and invasion.

Materials and Methods

Cell proliferation and viability assay.

$2x10^5$ B16F10 or THP-1 cells in their exponential growth phase were incubated in morphine-containing DMEM or RPMI respectively, supplemented with 10 % FBS and incubated at 5 % CO_2 / 37°C. Cell number and viability were evaluated every 24 hours for 3 consecutive days. Cell number and viability were measured using the hemocytometer and trypan blue, which selectively stains dead cells a dark blue. Cell viability is determine by the formula: Viability = 100% x the number of alive cells / total cells (non staining + blue stained cells).

DNA fragmentation assay.

To evaluate putative apoptosis induced by morphine, B16F10 and THP-1 cells were continuously grown in morphine-containing DMEM medium and assessed using the Apoptotic DNA ladder kit (Roche).

Invasion assay.

The conditions and apparatus used for the micro-chamber modified Boyden chamber assay have been previously described in detail (Basara *et al.*, 1985). Briefly, log-phase THP-1 and B16F10 cell cultured were harvested and resuspended in serum-free medium. The lower surface of polycarbonate filters were pre-coated with soluble fibronectin (SF) (1 μg of SF per ml in carbonate buffer). The filters were incubated overnight at 37°C. 2.5 x 10^5

cells in 250 µl of serum-free medium were placed in the upper chamber and 1 ml medium containing 10% FBS was placed in the lower chamber. For THP-1 cells, a filter with 5 µm pore size has been selected, while an 8 µm pore size was used for B16F10 cells. Chambers with cells were incubated for 7 hours at 37°C in humid 5% CO_2 atmosphere. After 7 hour of incubation, the filters were gently removed. Non-migrating cell in the upper well were removed using a cotton swab, followed by staining of the invading cells adhering to the lower surface of filter with Gentian Violet. Invasion was expressed as the median number of cells per high-power field that had crossed the fibronectin barrier and migrated to the lower surface of the filter, and was quantitated by counting the migrated cells in 5 randomly selected high-power fields per filter at a magnification of x 400. The results of triplicate assays are presented as medians and statistical analysis was evaluated using the Wilcoxon signed ranks. $P<0.05$ is considered statistically significant.

Cytoskeletal Actin.

2 x 10^4 cells were treated with RPMI or 1 µM morphine and placed on a BSA coated coverslip followed by incubation at 37°C in 5% CO_2 for 0.5 h - 5 h. Cells were then washed twice with PBS and fixed with a solution of 0.025% glutaraldehyde/0.25% formaldehyde for 10 min at 22°C. Cells were then washed 3 times with PBS, permeabilized with 0.5% Triton X-100 for 5 min and evaluated for F-actin using Alexa 488 phalloidin (Molecular Probes,

Eugene, OR). Samples were imaged using a quantitative confocal microscopy system (Meridian Instruments INC. Okemos. Michigan) coupled to an Olympus microscope and a 100x-oil immersion objective with a fixed pinhole setting of 40 μM.

Statistical analysis

Results are expressed as the median. Differences among groups were evaluated using a non-parametric Wilcoxon signed ranks test. $P < 0.05$ is considered to be statically significant.

Results

The effect of morphine on B16F10 and THP-1 proliferation and viability.

In order to examine the potential effect of morphine on B16F10 and THP-1 proliferation and viability, cells were cultured in media supplemented with 1 or 100 μM morphine. 1 μM morphine significantly inhibited B16F10 proliferation (Fig 3.1A), and decreased cell viability (Fig 3. 1B). In order to determine whether morphine induced apoptosis in these cells, camptothecin was used as a positive control. This reagent inhibits cell division and is a strong inducer of apoptosis. With the presence of camptothecin, in the medium, B16F10 proliferation and cell viability declined at 48 hours, and by 72 hrs no viable cells were detected. (Fig.3.1A and 3.1B). In the presence of 1 μM morphine THP-1 proliferation was not significantly affected. However, as the morphine concentration was increased to 100 μM morphine, cell proliferation was dramatically reduced (Fig 3.2).

Morphine does not induce apoptosis of B16F10 and THP-1 cells in vitro.

In order to test whether apoptosis contributed to the death of THP-1 or B16F10 cells treated with morphine, the analysis of DNA fragmentation in both cell types was evaluated. Although B16F10 cells showed apoptosis in response to camptothecin, morphine did not induce apoptosis in these cells in either 1%- or 10 %-FBS containing DMEM (Figure 3.3A). Morphine also failed to induce apoptosis in THP-1 cells(Figure 3.3B).

Morphine inhibits cytoskeletal actin polymerization in THP-1 but not in B16F10.

In order to evaluate this potential effect of morphine on cytoskeletal actin filaments in THP-1 and B16F10, cells were treated with 1 μM morphine for 20 minutes at 37°C followed by exposure of actin filaments to Alexa Fluor 488 phalloidin. Our observations show that morphine failed to reduce the F-actin content and induce cell shape changes in B16F10 cells. (Figure 3.4A). However, morphine reduced the content of polymerized actin filaments in THP-1 1 hour after exposure to morphine (Figure 3.4B).

Morphine inhibits B16F10 and THP-1 invasion across a fibronectin barrier.

Transwell Boyden Chambers were used to investigate the effect of morphine on invasion of THP-1 and B16F10 cells through a fibronectin barrier. In order to minimize the cytotoxicity of morphine to cells, this drug was used at concentration of 0.1, 0.3 and 1μM. Results demonstrate that at all concentrations morphine inhibited invasion of B16F10 across a fibronectin filter (Figure 3.5). 1 μM morphine also inhibited THP-1 invasion in the Boyden chamber. However, for THP-1 cells, the inhibition of invasion induced by morphine was dose-dependent, and 0.1 and 0.3 μM morphine did not significantly inhibit THP-1 invasion (Fig. 3. 6).

The inhibitory effect of morphine on B16F10 invasion is not associated with NOS activation, but is partially associated with N0S activation in THP-1 cells.

In order to evaluate the potential role of NO and opioid receptors in TC invasion inhibited by morphine, cells were pretreated with L-NAME, a NOS inhibitor and with naloxone, an antagonist of opioid receptors, prior to exposure to morphine. The results showed that the L-NAME failed to block the inhibitory effect of morphine on B16F10 invasion, but naloxone protected B16F10 from inhibition of invasion by morphine (Fig 3.7). The L-NAME-pretreated group showed no statistical difference when compared to the morphine-only treated group, demonstrating a NOS independent opioid dependent mode of action on inhibition of invasion of B16F10 cells. In contrast, pretreatment of THP-1 cells with L-NAME or naloxone significantly blocked but did not eliminate the inhibitory effect of morphine on invasion (Figure 3.8), demonstrating the partial involvement of NOS and opiate receptors in the morphine induced inhibition of THP-1 cells invasion.

Discussion

We found that morphine inhibited proliferation and exerted a cytotoxic affect on THP-1 and B16F10 cells. Our results showing decreased proliferation of THP-1 and B16F10 cells are consistent with previous reports that morphine inhibits proliferation in various cell types *in vitro* and *in vivo* (Fuchs *et al.*, 1993; Maneckjee *et al.*, 1994; Glasel *et al.*, 2000; Singhal *et al.*, 1998). However, our results extend this observation by showing that this cytotoxic affect appears to be independent of apoptosis. This suggests that morphine induces THP-1 and B16F10 death via a necrotic pathway. In these experiments, we also evaluated the effect of different concentrations of serum in the medium, which may affect cell proliferation and/or apoptosis, and found no statistically significant difference between 1% and 10% FBS.

It has been reported that opioids, such as morphine, induce apoptosis in lymphocytes and other types of cells. This opioid-induced apoptosis is mediated by the Fas-FasL signaling pathway (Yin *et al.*, 1999). The author suggests Fas (CD 95) expression induced by morphine appears to prime lymphocytes for elimination by apoptosis. In other words, the tightly regulated expression of Fas and FasL determines whether a lymphocyte survives or dies. This suggests that *in vitro* morphine may fail to induce apoptosis in THP-1 and B16F10 because of lack of co-expression of Fas and/or FasL in both cell lines. It is possible that morphine might be able to

induce B16F10 or THP-1 to undergo an apoptotic pathway *in vivo* if Fas were expressed on their cell surface and if FasL were available in the microenvironment.

It has been well demonstrated that cell shape is associated with diverse cell activities such as division, migration, adhesion and activation. Cell morphology is determined by the distribution and polymerization of cytoskeletal filaments including actin and microtubule filaments in cells. In this chapter, we have demonstrated that morphine either promotes depolymerization and/or inhibits polymerization of-actin in THP-1 resulting in a promotion of THP-1 rounding (Chapter 2). This observation is consistent with previous data showing that morphine induces cellular conformational changes in various types of cells including human monocytes, granulocytes and endothelial cells (Magazine *et al.*, 1996). The mechanism of morphine-induced cell rounding is associated with NOS activation and NO release. However, morphine failed to affect polymerization of actin in B16F10 cells, and this explains why this drug is not able to induce B16F10 cell rounding.

In this chapter, we also found morphine that morphine inhibits TC invasion across the fibronectin-coated filter in modified Boyden chambers (Transwell). In the invasion assay, cells travel across the fibronectin-coated filter on the down- side of membrane and must digest this coating in order

to penetrate fibronectin. The mechanism(s) of the morphine-induced inhibitory effect on B16F10 and THP-1 invasion were studied and compared. Morphine shows an inhibitory effect on TC invasion in both cell lines. THP-1 is less sensitive to morphine because only dose 1 μM morphine significantly inhibits invasion. However, B16F10 is extremely sensitive to morphine and 0.1, 0.3 and 1 μM morphine all significantly reduced B16F10 invasion. Naloxone, an antagonist of opioid receptors, partially protected the THP-1 cells and fully protected the B16F10 cells from a morphine induced inhibitory effect on invasion. In B16F10 cells the morphine-induced inhibition is mediated solely through activation of opioid receptors. However, L-NAME failed to block the morphine-induced inhibition in B16F10 cells, suggesting that NOS and NO are not involved the inhibitory mechanism induced by morphine in these melanoma cells. The ability of naloxone to block the effect of morphine in these cells, suggests the involvement of opioid receptors other than μ_3 in this cell line. This is consistent with our previous observation showing that morphine fails to induce NO production in B16F10 cells. The identification of this opioid receptor is not known. The identity of this receptor is important as it has obvious clinical relevance in view of its ability to regulate melanoma cell invasion. The fact that pretreatment with either nalaxone or L-NAME only partially prevented the morphine-induced inhibition of invasion in THP-1

cells may suggest an opioid receptor independent action of morphine on this cell type. Thus, morphine appears to be able to inhibit THP-1 invasion by a μ_3-NO mediated pathway, a μ_3 independent opioid receptor pathway, and/or an opioid receptor-independent pathway. Importantly, the results with SNAP demonstrate that NO can induce cell death in both cell types *in vitro*. Therefore, morphine-mediated NO release in leukocytes may contribute to the cause of morphine-indeed immunosuppression.

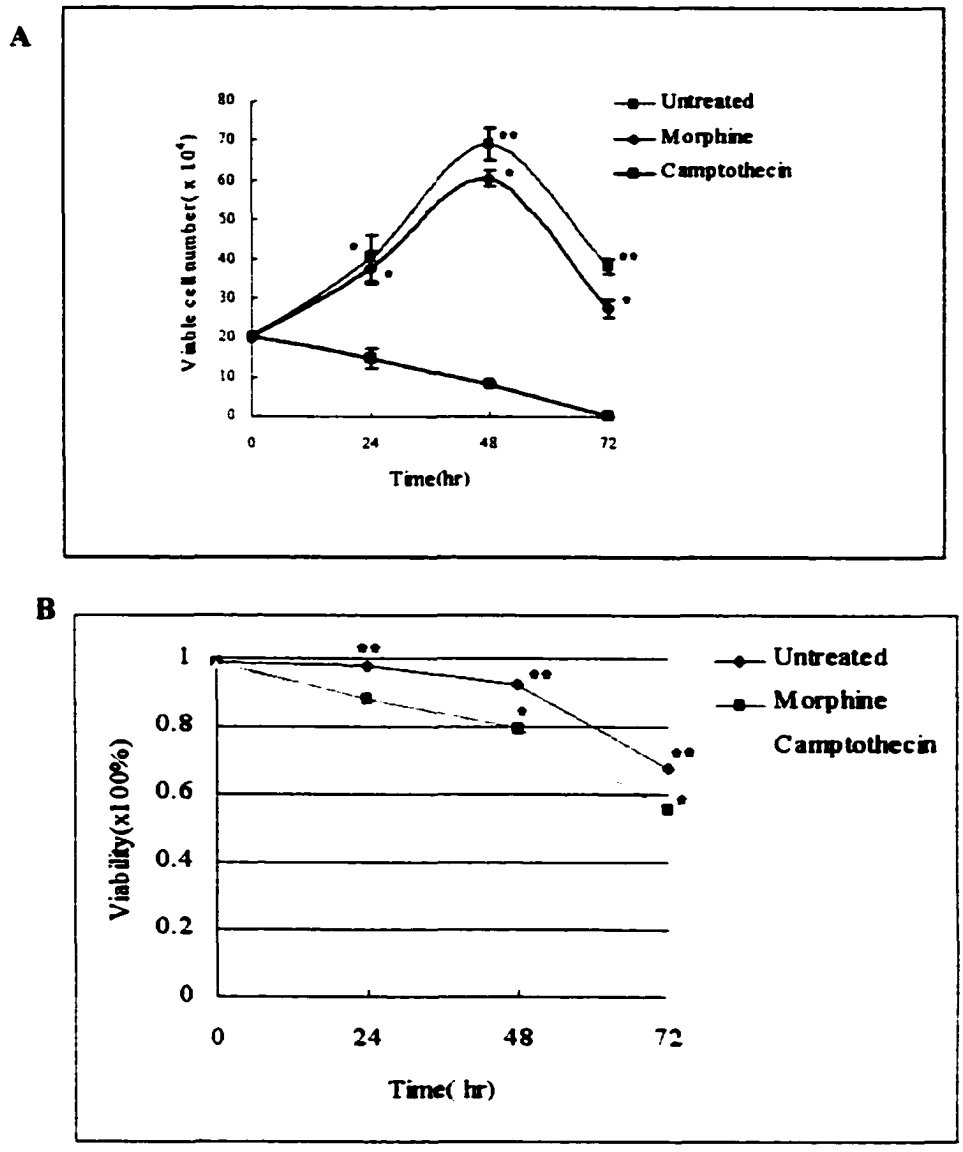


Figure 3.1A and 3.1B. Cell proliferation and viability of B16F10 in DMEM in the presence of morphine.

B16F10 cell were continuously cultured in DMEM containing 1 μM morphine, 4μg/mL Camptothecin or placebo and the viable cells were

measured at T 24-, 48- and 72- hour by using the hemocytometer method. Cells were harvested by using trypsin and centrifugation following by staining cells with trypan blue, which allows distinguishing viable cell from dead cells. The results indicated that morphine significantly inhibited B16F10 proliferation (** represented statistical significance of morphine-treated group to untreated and camptothecin-treated group, $P< 0.05$). Camptothecin completely inhibited B16F10 proliferation ($P<0.001$; * represented statistical significance of camptothecin-treated group when compared to the untreated and morphine-treated group.) (Figure 3.1A). Morphine induced a mild cytotoxic effect on B16F10 cells ($P< 0.05$; ** represents statistical significance of morphine-treated group to untreated),(Fig 3.1B) and camptothecin exerted extreme cytotoxicity on B16F10 cells ($P<0.001$; * represented statistical significance of the camptothecin-treated group when compared to the untreated), (Fig.3.1B). Data are represented as medians obtained from triplicate experiments. The cell proliferation and viability for each group at T 24 and T48 were analyzed for statistical significance using the Wilcoxon signed ranks test.

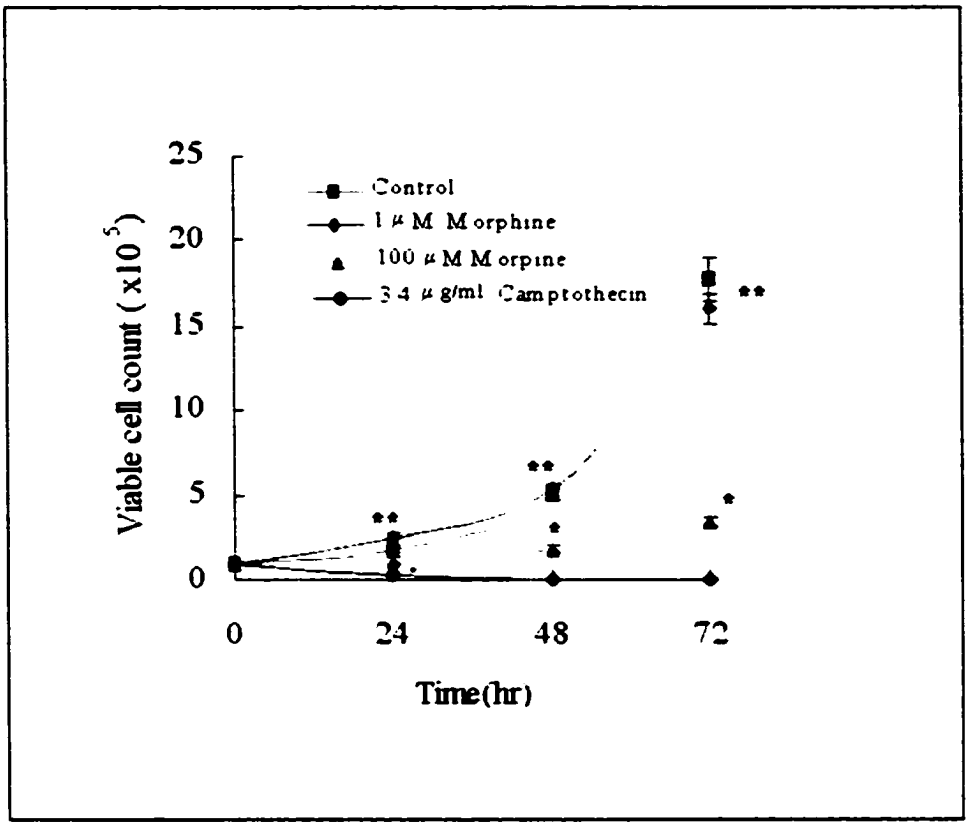
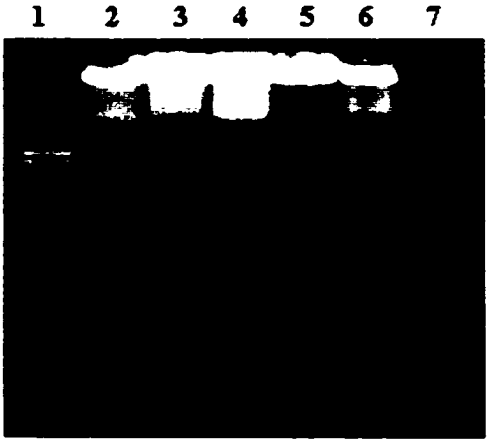


Figure 3.2. The effect of morphine on THP-1 proliferation.

1 x10^5 THP-1cells grew in 10 % FBS containing RPMI supplemented with morphine at concentration of 1 and 100 μM, or camptothecin. The measurement of viable cell number was performed at T24, T48 and T72 after cells were cultured. The cell proliferation of THP-1 cultured in 1 μM morphine was slightly declined at T72. However, increased morphine concentration of 100 μM, THP-1 cell proliferation is significantly declined

(P< 0.05; * represented statistical significance of 100 μM morphine-treated group to other groups). Camptothecin absolutely inhibits THP1 growth in RPMI (P<0.05; ** represented statistical significance of camptothecin-treated group to other groups). Results are represented as median resulted from three different experiments and the statistical significance was evaluated by using Wilcoxon signed ranks analysis.



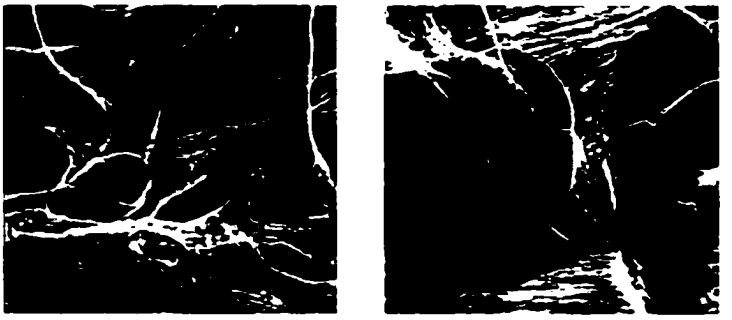
A.

B.

Figure 3.3A and 3.3B. The analysis of DNA fragmentation of B16F10 and THP-1 induced by morphine.

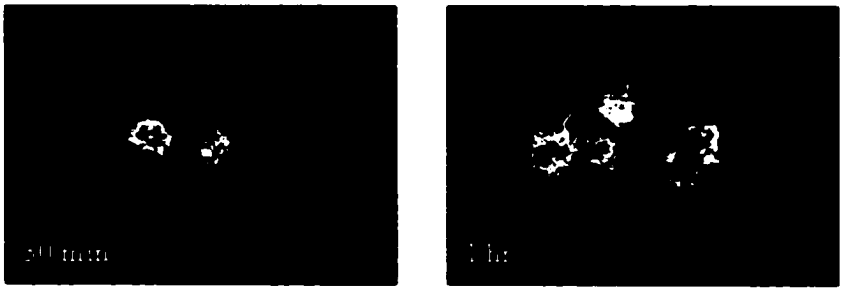
80 % confluent B16F10 cells were treated continuously with 1, 100 μM morphine or 3.4μg/ml camptothecin, in DMEM at 37°C for 24 hours before analysis. Preparation of DNA extract and analysis of DNA fragmentation

analysis was performed as described in the section of method and material. Lane 1: DNA marker. Lane 2: Cells grew with 100μM-morphine-containing medium supplemented with 1%FBS. Lane 3: Cells grew with 1μM-morphine-containing medium supplemented with 1%FBS. Lane 4: Cells grew in RPMI medium only Lane 5 Cells grew in 1 μM-morphine-containing medium supplemented with 10%FBS. Lane 6 Cells grew with 100μM-morphine-containing medium supplemented with 10%FBS. Lane 7. Cells grew in camptothecin-containing RPMI for 12 hours (Fig.3.3A). THP-1 Cells were treated continuously with 1 or 100μM morphine or 3.4μg/ml camptothecin in RPMI at 37°C for 6 hours before analysis. Lane 1: DNA marker. Lane 2: Cells were cultured in medium only. Lane 3: Cells grew in medium supplemented with 1 μM morphine. Lane 4. Cells grew in medium supplemented with 100 μM morphine. Lane 5: Cells grew in camptothecin-containing medium. Line 6: A positive control (Fig.3.3B). (Supplied by Roach, apoptotic DNA sample induced by camptothecin).



Untreated Morphine

A.



Untreated

Morphine

B.

Figure 3.4.A and 3.4B. Changes in morphology and cytoskeletal actin filaments of B16F10 (A) and THP-1 following exposure to morphine (B).

B16F10 and THP-1 cells were prepared and processed as described in the material and methods. The polymerized F-actin was stained using Alexa 488 phalodin and evaluated by confocal microscopy. The cell was optically sectioned and fluorescence was determined using a 100X objective and a pinhole setting of 40 μM. Figure 3.4A. The pattern and content of cytoskeletal actin (F actin) of B16F10 have no significant change after exposure to morphine in two hours. Figure 3.4B. Morphine reduces the formation of F-actin of THP-1 in 0.5 and 1 hour after exposure to morphine. This experiment has been carried out for 4 times, and each time at least 5 slides for each group has been evaluated.

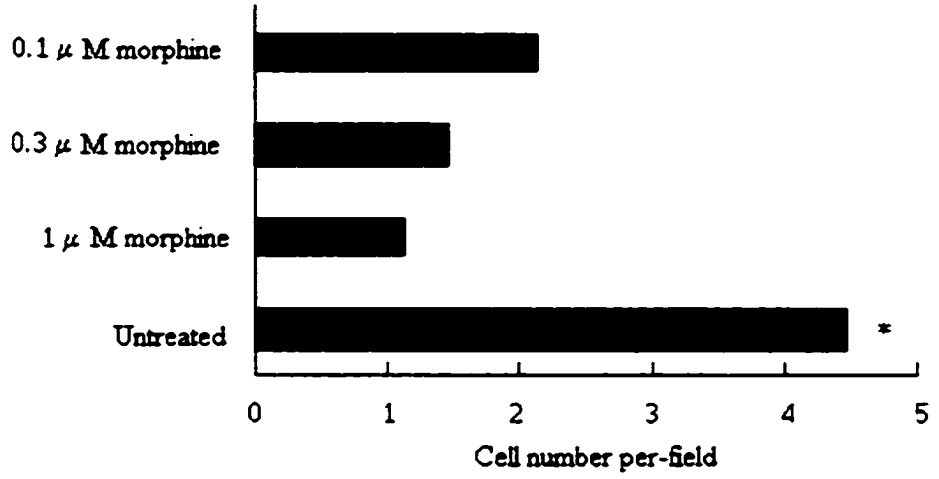


Figure 3.5. Inhibition of B16F10 invasion by morphine.

250 μl cell suspension containing 2 x 10^5 B16F10 cells was loaded to the upper well of Boyden chamber and incubated with various concentration of morphine from 0.1, 0.3 and 1 μM respectively for 7 h at 37°C. The cell invasion was quantified by counting the cells in 5 random medium-power field (x 400) on the lower surface of filters from 3 replicate Transwell chambers. The results indicated that all concentrations of morphine inhibited B16F10 invasion. There is no statistically significant difference among 0.1, 0.3 and 1 μM morphine on B16F10 invasion. *Denotes statistical significance; $P< 0.05$, when compared to morphine treated cells. The cell invasion was analyzed for statistical significance by using Wilcoxon signed ranks test.

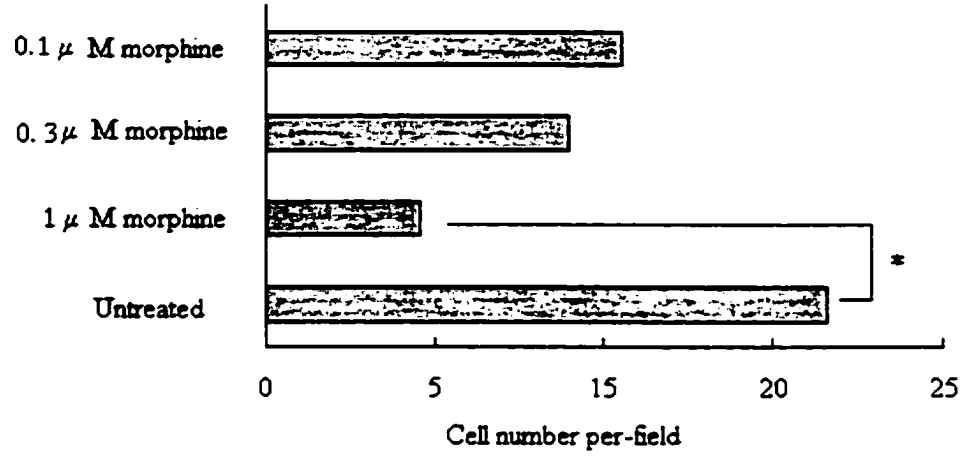


Figure 3.6. Inhibition of THP-1 invasion by morphine.

The method for this experiment was performed as same as previously described in Fig.5. In this experiment, RPMI medium was used for culturing THP-1, instead of DMEM. 1 μM Morphine significantly inhibited THP-1 invasion. However, 0,1 and 0.3 μM morphine did not significantly inhibit THP-1 migration across the fibronectin-coated filter. Data expressed as median and *denotes statistical significance; $P< 0.001$, when compared all other groups. The cell invasion was analyzed for statistical significance by using Wilcoxon signed ranks test.

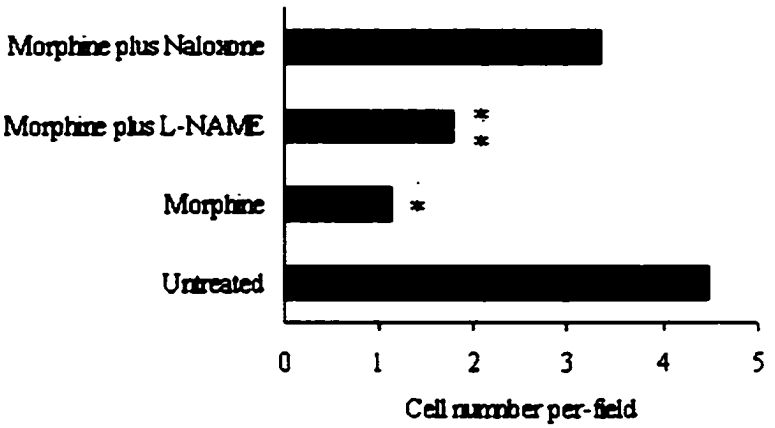


Figure 3.7. The response of B16F10 to L-NAME and Naloxone in invasion inhibited by 1 μ morphine.

B16F10 cells were pre-treated with 100 μM of L-NAME, 10 μM naloxone or vehicle for 20 minutes prior to exposure to morphine in upper well. Cell invasion was measured by the method as previously described in Figure 3.5. L-NAME failed to block the inhibitory effect of morphine on B16F10 invasion. However, naloxone significantly suppressed the inhibition of B16F10 invasion induced by morphine. Data expressed as a median of cell count per field. * and ** both denote statistical significance; $P< 0.001$, when compared with the untreated and naloxone-plus-morphine groups. Cell invasion was analyzed for statistical significance using the Wilcoxon signed ranks test.

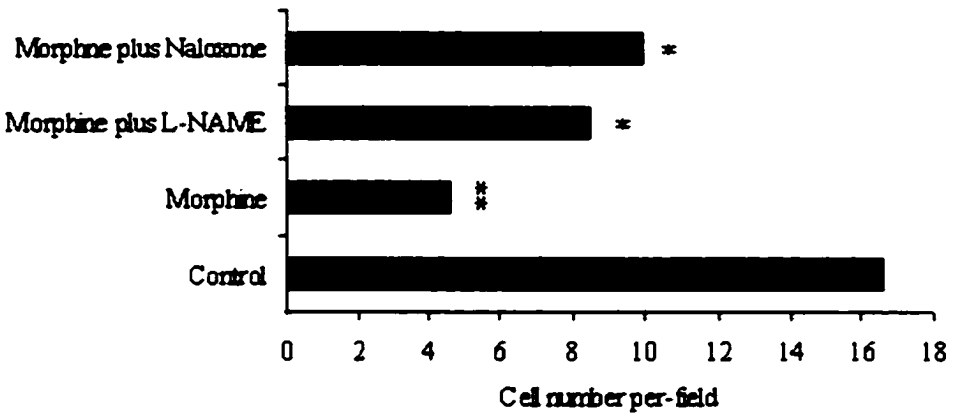


Figure 3.8. The response of THP-1 to L-NAME and Naloxone in invasion inhibited by morphine.

Prior to incubate cells with morphine, THP-1 were pretreated with L-NAME, naloxone or vehicle. Cell migration was evaluated as described in Fig 3.5. L-NAME and Naloxone only partially blocked the inhibitory effect of morphine on THP-1 invasion. Data expressed as a median of cell count per field. **P< 0.001, when compared to the untreated, L-NAME- and naloxone-pretreated groups. *P < 0.05, compared to untreated. The cell invasion was analyzed for statistical significance using the Wilcoxon signed ranks test.

CHAPTER FOUR

VASCULAR ADHESION OF B16F10 MELANOMA IS INCREASED FOLLOWING *IN VITRO* OR *IN VIVO* EXPOSURE OF THE AORTA TO MORPHINE

Introduction

Metastasis is an intricate process that involves the successful translocation and proliferation of tumor cells (TC) within the host. Several steps in TC metastasis have been identified and include escape from the primary tumor, penetration and migration through the basement membrane, entry into the vascular or lymphatic circulation, arrest within the microcirculation of distant organs followed by extravasation and proliferation (Fidler *et al.*, 1978; Poste *et al.*, 1980; Liotta *et al.*, 1983). The interaction of TC and blood-borne tumor emboli with the vascular endothelium is a critical event in the metastatic process prior to extravasation. Although the endothelium has been demonstrated to have barrier function, limiting TC access to the basement membrane, a myriad of endogenous pro-inflammatory factors such as interferon gamma (Scher *et al.*, 1993), thrombin (Wojtukiewicz *et al.*, 1992;

1993 and 1995), interleukin-1 (Stefani *et al.*, 1999; Rice *et al.*, 1988; Chirivi *et al.* 1993; Cohen *et al.*, 1999), tumor necrosis factor (Moser *et al.*, 1989), eicosanoid metabolites (Tang *et al.*, 1994) and lipopolysaccharides (Kalogeris *et al.*, 1999) have been reported to promote TC adhesion and facilitate metastasis. Therefore, factors that alter TC – vascular interaction or integrity of the vascular endothelium are likely to alter tumor metastatic potential.

The favorable analgesic properties of morphine are well known accounting for its widespread use for postoperative and intractable pain management (Fricchione *et al.*, 1994; Stefano et al., 1994). Despite its frequent use in patients with cancer, the direct effects of morphine on TC adhesion and metastasis have not been well studied. Morphine triggers nitric oxide (NO) release from rat and human vascular endothelium and modulates inflammatory cell adhesion (Stefano *et al.*, 1995; King *et al.*, 1997). However, the potential biological impact of morphine-coupled NO release on metastasis is complex and difficult to predict due to activation of multiple events with opposing action. Nitric oxide release from the vascular endothelium could attenuate TC–vascular interaction and adhesion (Kong *et al.*, 1996) resulting in reduced metastasis. Alternatively, a morphine-induced rounding of human arterial endothelial cells (Magazine *et al.*, 1996) suggests that morphine could facilitate TC – vascular interaction as a result of increased exposure of underlying extracellular matrix. In the present study

we used B16F10 melanoma cells and mouse aorta as a model for TC-vascular interactions, and explored the effect of morphine exposure *in vitro* and *in vivo* on TC adhesion.

Material and Methods

Animals

C57BL/6 mice (Charles River, Ballardville Mass), and C57BL/6.129P2-NOS3^{tm1Unc} mice (Jackson Laboratories, Jacksonville, Florida) were 8 – 12 weeks of age when used in experiments.

Melanoma cell preparation

The murine B16F10 melanoma was obtained from Dr. Szalay. Cells were grown in DMEM (Gibco, Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS), 0.1mg/ml streptomycin, 100 U/ml penicillin G and maintained at 5 % CO_2 / 37°C. Cells were harvested using trypsin, 0.25% and incubated with 10 μM Cell Tracker (Molecular Probe) for 40 min at 37°C to fluorescently-label the cells. Cell Tracker labels only live cells and does not alter B16F10 vascular adhesion or metastasis (Szalay *et al.*, 2001). Unincorporated dye was removed by extensive washing with DMEM whereupon the cells were resuspended in fresh DMEM and maintained at 37°C prior to use in assays.

Nitric Oxide Detection

For evaluation of NO release from vascular preparations, mice were sacrificed by intraperitoneal injection with 0.25 ml pentobarbital sodium (120mg/kg) followed by rapid excision of the thoracic aorta. The vessels

were placed in ice-cold PSS (130 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 14.9 mM $NaHCO_3$, 5.5 mM Dextrose, 0.03mM EDTA, and 2.5 mM $CaCl_2$), pH 7.4 and excess connective and adipose tissue were removed. The aorta was cut into 2- to 3-mm segments and placed in 5 ml of aerated (95%O_2-5%CO_2) PSS maintained at 24°C for evaluation of NO release in real time using a NO-selective amperometric probe exactly as previously described (Magazine *et al.*, 1995). Briefly, the probe was allowed to equilibrate for 10 min with the tissue to establish a baseline reading followed by addition of morphine and measurement of NO levels for an additional 45 min. To evaluate the specificity of the response to morphine *in vitro*, tissue was pretreated with the NOS inhibitor, L-NAME (10 μM) or the morphine receptor antagonist, naloxone (10 μM) for 10 min prior to addition of morphine. For statistical evaluation, peak levels of NO release in N=5 experiments was recorded and evaluated for differences using the Wilcoxon non-parametric test. Aortic segments denuded of endothelium were prepared by insertion of a smooth metal wire into the lumen of the vessel followed by gentle scraping of the tissue as described (King *et al.*, 1997). For evaluation of NO release from TC, B16F10 cells ($1x10^6$) were resuspended in 1 ml PSS and NO release in response to treatment with 1 μM morphine was evaluated exactly as described for aorta.

Melanoma adhesion to the vascular endothelium

Aortic segments were excised from mice and prepared for use exactly as described above for NO determination except that prepared tissue was placed in DMEM and maintained at 37°C, 5 % CO_2 for the duration of the assay. The aortic ring was cut, exposing the luminal surface and a notch was created in the upper left corner to facilitate orientation of the tissue upon conclusion of the assay. The aorta was treated with buffer or 1 μM morphine for 20 minutes prior to addition of the cell-tracker-labeled B16F10 cells ($5x10^4$ cells/ml) for 60 min. Aortic segments were rinsed with pre-warmed PSS to remove non-adherent cells whereupon the tissue was fixed with 3.7% formaldehyde for 15 minutes at room temperature, placed on a slide with the luminal surface up and mounted with a coverslip using gel-mount media. B16F10 adhesion to the luminal surface of the aorta was evaluated using quantitative fluorescent confocal microscopy and a Meridian Ultima confocal microscope. Tissue was visualized using a 40x oil immersion lens and 5 random fields of view were evaluated per aorta. For quantitation of B16F10 adhesion, the PMT detector was set a level where only the cell-tracker-labeled B16F10 cells were visualized and autofluorescense of the tissue was negated. The pinhole setting on the confocal microscope was set to 100 um so that only the luminal surface was visualized. For experiments that evaluated *in vivo* exposure to morphine, mice where injected IP with 5 mg/kg

morphine, saline or L-NAME only, or L-NAME followed 30 minutes later by morphine. This was done by Jennifer Adjodha. Mice were then sacrificed and the tissue was processed exactly as described above for *in vitro* studies.

To evaluate the direct effects of NO on TC adhesion, experiments were performed exactly as described above except that aorta was treated with the NO donor, S-Nitroso-N-acetyl-D, L-peniciliamine (SNAP), 1 μM for 10 min and washed extensively prior to exposure to TC. To evaluate the effects of integrin recognition on TC adhesion, cells were treated with RGDS or RGES, 0.3 to 1.0 mM for 10 min prior to exposure to vascular tissue and the experiments were completed exactly as described above.

Results

Morphine induces an EC-NOS dependent release of NO from aortic rings.

Mouse aorta was exposed to morphine and NO release was evaluated in real-time using an NO-selective amperometric probe exactly as described (King *et al.*, 1997). Exposure of mouse aortic rings to morphine triggered a rapid release of NO that was maximal within 5 min and declined to baseline levels within 10-15 min (Fig 4.1). Morphine induction of NO release was abrogated in the presence of the NOS inhibitor, N^{w}-nitro-L-arginine methyl ester (L-NAME, Fig 4.1B and 1C), the opiate-alkaloid receptor antagonist naloxone, or in rings denuded of endothelium (Fig 4.1C) The rapid and transient release of NO induced by morphine is consistent with ecNOS activation (Stefano *et al.*, 1995) and with previous studies that demonstrate morphine induction of NO release by an L-arginine, endothelium-dependent pathway in rat and human endothelium (Magazine *et al.*, 1996).

Morphine fails to induce NO release form endothelium of ecNOS knockout mice.

To confirm that NO released from endothelium stimulated by morphine required ecNOS activation, experiments were repeated using aortic segments from the ecNOS knockout mouse, C57BL/6.129P2-NOS3^{tm1Unc}. In contrast to the aorta from C57BL/6 mice, morphine failed to trigger a significant increase in NO release from C57BL/6.129P2-NOS3^{tm1Unc} aorta (Fig. 4.2A).

These data, taken together, suggest that morphine stimulates NO release from mouse aortic endothelium by an ecNOS dependent mechanism. The contribution of TC NO release in response to morphine was evaluated. In contrast to C57BL/6 aorta, morphine did not induce significant NO release from B16F10 cells (Fig. 4. 2B).

Morphine Increases Adhesion of TC to the aorta and adhesion is EC and NOS III dependent.

Adhesion of B16F10 cells to the luminal surface of mouse aorta was evaluated in the presence or absence of morphine. Adhesion of B16F10 cells to mouse aorta in vitro (Fig. 4.3A) was increased markedly following exposure of the aorta to morphine (Fig. 4.3B). The significant increase in TC adhesion observed in the presence of morphine was reduced to control levels by pretreatment with L-NAME or naloxone. Treatment of aorta with L-NAME or naloxone alone did not have a significant impact on B16F10 adhesion (Fig. 4.4).

Morphine-induced TC adhesion required the presence of the endothelium on aortic rings.

To evaluate the role of vascular endothelium in morphine mediated TC adhesion. experiments were repeated as above using aorta denuded of endothelium. B16F10 adhesion was increased significantly in aorta denuded of endothelium relative to that of endothelium-intact controls (Fig. 4.5).

However, in contrast to intact aorta, exposure of aorta denuded of endothelium to morphine failed to increase TC adhesion.

TC adhesion induced by morphine required the activation of ecNOS

To evaluate the role of NOS III in morphine mediated TC adhesion, experiments were performed as described above using aorta of the NOS III knockout mouse, C57BL/6.129P2-NOS3^{tm1Unc}. B16F10 adhesion to aorta of NOS III knockout mice was not increased in the presence of morphine (Fig. 4.6). In contrast, B16F10 adhesion was increased significantly in rings pretreated with the NO donor, SNAP. These data taken together demonstrate that exposure of NOS III knockout aorta to NO is sufficient to mimic the effects of morphine observed in C57BL/6 mice (Fig. 4. 3) and that NOS III is required for morphine-induced increase in TC adhesion.

The morphine-induced increase in adhesion of TC to the endothelium is abrogated by RGDS peptide.

Exposure of TC to 100 or 300 μM RGDS peptide prior to addition of the TC to aortic ring preparations resulted in decreased adhesion to the vascular endothelium of intact or endothelium denuded untreated aorta when compared to TC not exposed to RGDS and adhesion was almost completely abolished by 300 μM of peptide (Figure 4.7A). We therefore used 300 μM in subsequent experiments. Treatment of aortic rings with morphine failed to increase adhesion of RGDS-treated TC, and an RGDS inhibition of TC

adhesion comparable to that observed in the absence of morphine was observed (Figure 4.7B). Treatment of TC with the control peptide RGES, had no significant impact on TC adhesion (not shown).

In vivo exposure of mice to morphine increases TC adhesion to aortic rings.

The effects of morphine on TC adhesion *in vitro* may not correlate with the effects of morphine observed *in vivo*. To assess the effects of *in vivo* exposure to morphine on TC adhesion, experiments were performed as above except that C57BL/6 mice were exposed to morphine *in vivo* for 1 hour prior to animal sacrifice, excision of the aorta and evaluation of B16F10 adhesion *in vitro*. Adhesion of B16F10 to aortic segments of mice exposed to morphine *in vivo* was markedly increased relative to untreated controls (Fig. 4.8). Treatment of mice with L-NAME 30 min prior to injection with morphine abrogated the increased adhesion of B16F10 to aortic segments *in vitro*.

Discussion

Nitric oxide has been demonstrated to alter tumor metastatic capability, differentiation and viability (Stuehr *et al.*, 1989; Farias-Eisner *et al.*, 1994; Jadeski *et al.*, 1999; Lala et al., 1998; Wink *et al.*, 1998; Li et al., 1991; Wong *et al.*, 2000; Pipili-Synetos *et al.*, 1995; Umansky *et al.*, 1996). Various cancers have been demonstrated to produce NO, and NO release has been correlated with both increased and decreased tumor growth and metastasis (Jadeski *et al.*, 2000; Joshi et al., 1996; Reveneau *et al*, 1999; Shi *et al.*, 2000; Xie *et al.*, 1996; Edwards *et al.*, 1996; Duenas-Gonzalez *et al.*, 1997). Morphine stimulates release of NO from rat and human vascular endothelium following activation of the μ3 morphine-selective, opioid peptide insensitive receptor by a μ3-dependent mechanism in rat and human vascular endothelium (Stefano *et al.*, 1995). In addition morphine has been demonstrated to potently induce rounding of human arterial endothelial cells (Magazine *et al.*, 1996), inhibit the expression of some cellular adhesion molecules on vascular endothelium (Stefano *et al.*, 1998) and inhibit immune responsiveness (Bryant *et al.*, 1990; Sibinga *et al.*, 1988; Gomez-Flores *et al.*, 1999; Beilin *et al.*, 1989; Welters *et al.*, 2000). These data when taken together suggest that morphine-coupled NO release may impact tumor metastasis.

Adhesion of TC to the endothelial layer, or to the underlying sub-endothelium is considered to be a critical determinant of metastatic potential. In the present study, mouse aorta was employed as a model to evaluate morphine-mediated changes in TC–vascular endothelium interactions. Stimulation of aortic rings with morphine followed by exposure to B16F10 cells resulted in a significant increase in B16F10 adhesion to the luminal surface of the aortic tissue 1-2 hours later. As expected, a significant increase in NO release was observed following morphine stimulation of the mouse aorta *in vitro* (Fig. 4.1A). This release was rapid, transient and abrogated in the presence of the NOS inhibitor, L-NAME, or the opiate receptor antagonist, naloxone, consistent with cNOS activation (Magazine *et al.*, 1995). The failure of morphine-treated B16F10 cells to release NO simplified our studies by demonstrating that morphine-induced NO release in our system was restricted to the aorta. This is consistent with our findings that stimulation of TC with morphine had no effect on TC adhesion to aortic rings (unpublished data).

To further evaluate the role of the endothelium and ecNOS, the effect of morphine on B16F10 adhesion was examined using aorta from NOS III knockout mice. Morphine failed to induce significant NO release from NOS III knockout aorta suggesting that morphine requires ecNOS expression to induce NO release. Importantly, morphine treatment of aortic segments from

these mice did not increase B16F10 adhesion suggesting that μ3 receptor activation is necessary but not sufficient to increase melanoma adhesion; ecNOS coupled NO release is also required.

A variety of CAMs have been demonstrated on melanoma cells and include LFA-3, VLA-4, VCAM-1, ICAM-1, as well as β1 and β_3 integrins (Pandolfi *et al.*, 1992, Rice *et al.*, 1989; Grossi *et al.*, 1988; Chopra *et al.*, 1991; Martin-Padura *et al.*, 1991). Increased expression of the $\alpha 11b\beta_3$ integrin in B16 melanoma cells has been shown to increase adhesion to EC and sub-endothelial matrix (Grossi *et al.*, 1988) and B16a cells have been shown to possess a large intracellular pool of alpha $11b\beta_3$ that can be mobilized to the cell surface by appropriate stimuli (Chopra *et al.*, 1991). In our experiments, adhesion of B16F10 melanoma cells to the intact aorta was ablated in the presence of 1 mM RGDS yet was unaffected by 1 mM RGES, demonstrating that B16F10 melanoma cell integrins play a major role in mediating adhesion to aortic endothelial cells (Martin-Padura *et al.*, 1991). Previous studies have demonstrated that cellular adhesion is markedly attenuated by endothelial cell NO release (King *et al.*, 1997; Kong et *al.*, 1996; Stefano *et al.*, 1998). However, this attenuation of adhesion by NO was transient lasting only 10-30 minutes, and slower, longer lasting affects were also observed. Adhesion of monocytes to human saphenous vein was reduced 10-30 min following morphine treatment yet increased to a level

equal to or greater than observed in untreated vessels 2-5 hours post morphine treatment; an effect not observed in tissue denuded of endothelium (Stefano *et al.*, 1998). Furthermore, treatment of cultured endothelial cells with morphine also induced changes in cell conformation that were slow and prolonged.

During metastasis of solid tumors, blood-borne tumor cells (TC) must recognize and adhere to the endothelium of blood vessels, leave the vessel and colonize a target organ. TC attachment to endothelial cells (EC) is widely considered to be a critical step in metastasis. Thus, it is significant to note that morphine is capable of altering adhesion to the vascular endothelium. There are several possible explanations for morphine's ability to increase adhesion of the melanoma cell to the EC. Morphine may induce conformational changes in endothelial cells resulting in increased exposure of the subendothelial matrix. Such a mechanism would explain the inability of morphine to increase TC adhesion in vessels denuded of endothelium since matrix exposure is already at maximum. Alternatively, increased TC adhesion to the aorta may be mediated by a morphine-induction of TC integrin expression. We have found that pretreatment of B16F10 cells with morphine followed by extensive washing prior to addition to the aorta did not result in an increase in TC adhesion (data not shown), suggesting that morphine does not directly induce B16F10 CAM expression. However, NO

activation of the EC may result in the release from this cell of one or more substances capable of increasing melanoma cell CAM expression. In addition, since the time course of reaction of different cell types to morphine is variable and little is known regarding the time course of morphine-induced alterations of CAM expression in mouse aortic EC, it is possible that following an expected initial decrease in EC CAM expression, a subsequent increased expression of EC-CAM may occur and be manifest 1-2 hours later.

In summary, our in vitro data demonstrate that morphine treatment can induce an EC-NOS and NO mediated alteration of TC-vascular interaction, resulting in increased TC adhesion to the vessels 1-2 hours after drug exposure. This morphine induced alteration in TC-EC adhesion at least in part, is mediated by melanoma cell surface integrins. The potential in vivo effect of morphine on TC adhesion to the EC is demonstrated by the observation that administration of an analgesic dose of morphine impacted subsequent in vitro adhesion of TC to the vascular endothelium. These data taken together suggest that morphine may have a systemic effect of TC adhesion. Extrapolation of these results to the microvasculature *in vivo* suggests that morphine administration could have profound effects on melanoma metastasis in cancer patients.

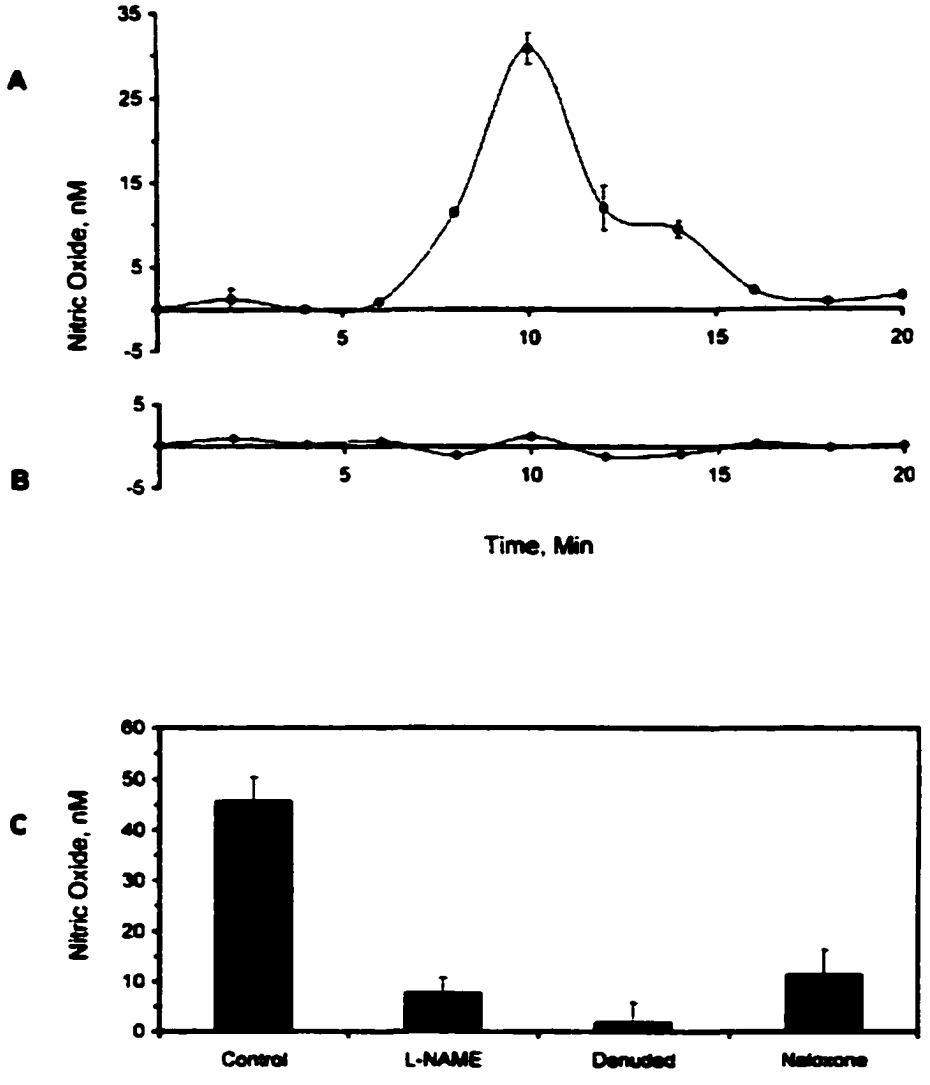


Figure 4.1. Evaluation of NO production by mouse aorta.

Mouse aortic rings were treated with 1 μM morphine and NO release was evaluated in real-time using a NO-selective amperometric probe (Word

Precision instruments, FL). Stimulation of aortic rings with **A.**, morphine rapidly induced an increase in NO production that was blocked by **B.**, pretreatment of the rings with 10 μM L-NAME. Morphine-induced NO release was evaluated as described above and maximal release of NO in response to morphine is shown in **C.** Morphine-induced NO release was significantly reduced by pre-treatment with L-NAME, naloxone or by denudation of endothelium. The data show is the mean ± SD of N=5 experiments.

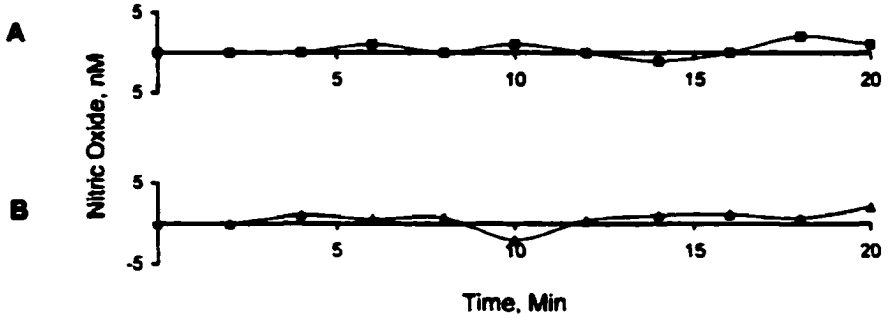


Figure 4.2. Evaluation of NO production from C57BL/6.129P2-NOS3^{tm1Unc} and B16F10 TC.

Nitric oxide release was evaluated as described in Figure 1A above. A. Morphine failed to trigger the NO release from the aorta obtained from NOS III gene knockout mice. B. Morphine failed to induce the NO release from B16F10 cells. The data show is the mean ± SD of N=5 experiments.

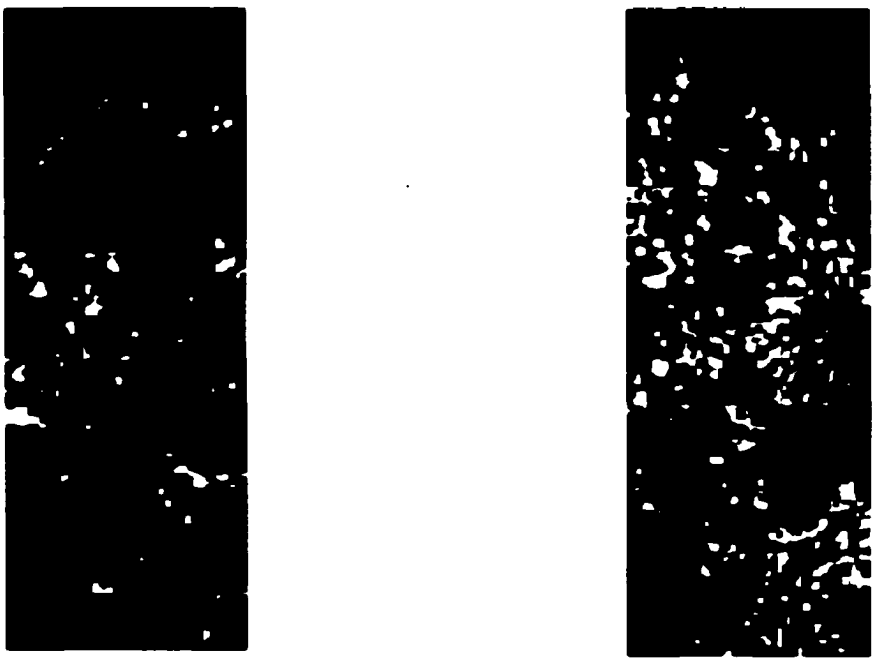


Figure 4.3. B16F10 adhesion to the mouse aorta.

Mouse aorta was treated with morphine 20 min prior to introduction of cell-tracker labeled B16F10 cells for 60 min whereupon adhesion to the luminal surface was evaluated using quantitative fluorescent confocal microscopy. The photomicrograph shown is representative of n=8 experiments that all demonstrated a marked increase in B16F10 adhesion following treatment of aorta rings with morphine.

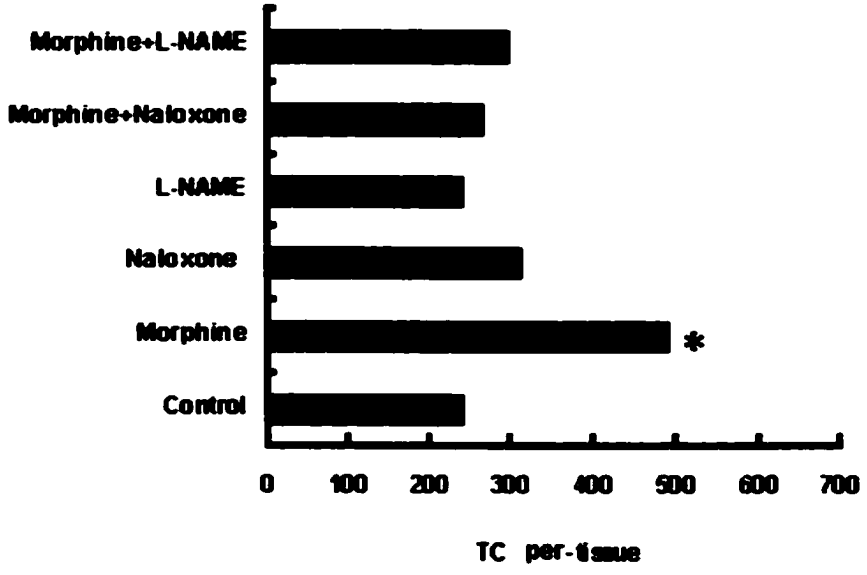


Figure 4.4. Augmented adhesion of B16F10 TC to aorta by morphine.

Experiments were performed as described in Figure 4.3 except that aortic rings were pretreated with the morphine receptor antagonist, naloxone (10μM) or the NOS inhibitor, L-NAME (100 μM) prior to exposure to morphine (1μM). Pretreatment of rings with naloxone or L-NAME alone did not alter adhesion of tumor cells to aortic rings. Treatment of aortic rings with morphine resulted in a significant increase in tumor cell adhesion (*$P < 0.001$; denotes statistical significance in adhesion between morphine with all other group, (N= 8). The data are represented as medians and cell adhesion was analyzed for statistical significance using the Wilcoxon signed ranks test.

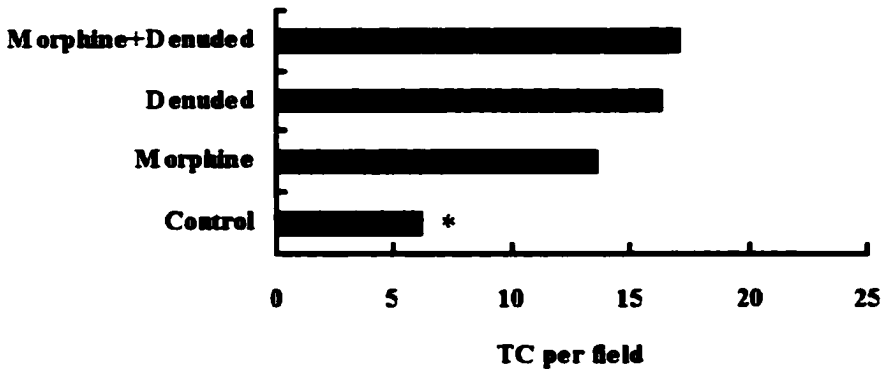


Figure 4.5. B16F10 adhesion to the denuded mouse aorta.

Experiments were performed as described in Figure 4.3 except that aorta was denuded of endothelium. Adhesion of TC to aorta denuded of endothelium was significantly increased relative to that of intact controls (*P < 0.001, *denotes statistical significance between control and all other groups). The retention of TC to the luminal surface of aorta denuded of endothelium was not increased following treatment with morphine. Data shown are the medians and N=7 experiments. The cell adhesion was analyzed for statistical significance using the Wilcoxon signed rank test.

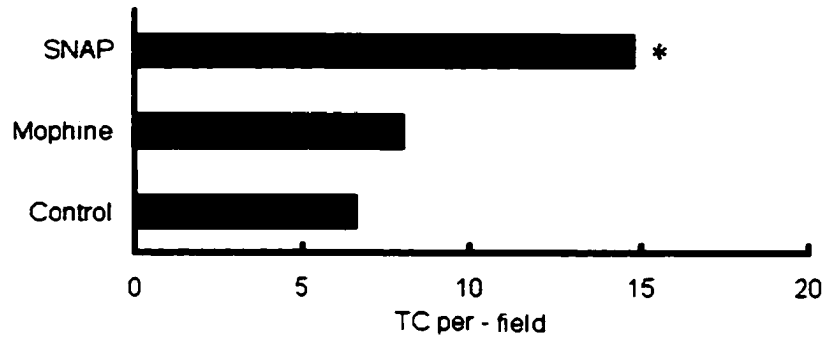


Figure 4.6. B16F10 cell adhesion to the aorta from the ecNOS Knockout mouse.

Experiments were performed as described in Figure 4.3 except that the aorta was from the NOS III knockout mouse (C57BL/6.129P2-NOS3^{tm1Unc}). Morphine failed to increase TC retention to aorta from NOS III knockout mice. In contrast, treatment of the aorta with the NO donor, SNAP resulted in a significant increase in TC retention (*P < 0.01; denotes statistical significance in SNAP treatment compared to other groups.) that was similar to the effect of morphine on C57Bl/6 aorta. (The data shown are the medians of N=4 experiments).

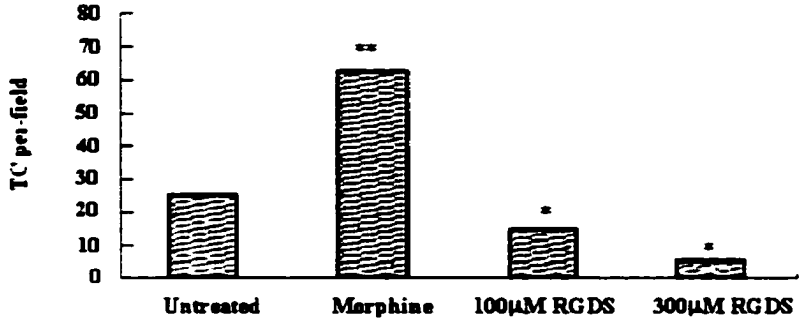


Figure 4.7A. The RGDS peptide inhibits B16F10 adhesion to aorta.

The experiments were performed exactly as described in Figure 3 except that TC were exposed to RGDS peptides prior to exposure to the aorta. The adhesion of B16F10 cells to aorta were significantly reduced, when cells were pretreated RGDS either at 100 μM and 300μM. (*Denotes statistical significance in RGDS treatment with untreated and morphine treated *P < 0.01, analyzed by Wilcoxon signed rank statistical analysis and **denotes statistical significance in morphine treatment compared to untreated and the RGDS pretreated group; P < 0.01, statistical significance were analyzed by Wilcoxon signed rank test). The data shown are the medians of N=3 experiments.

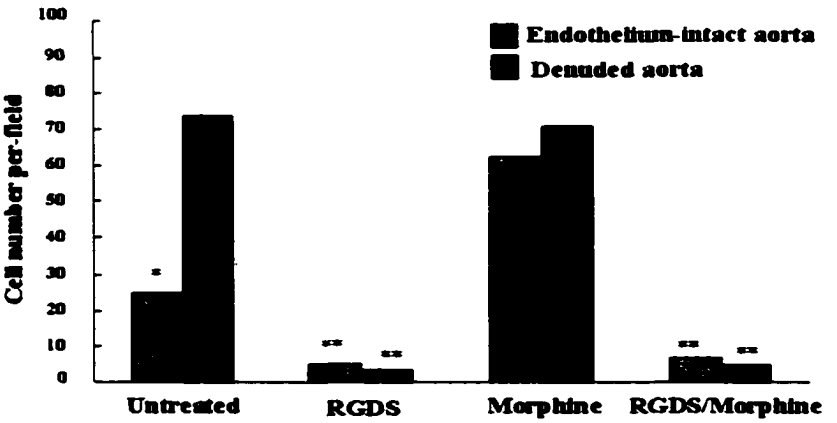


Figure 4.7B. Integrin-mediated B16F10 adhesion to the aorta.

The experiments were performed exactly as described in Figure 3 except that TC were exposed to RGDS peptides prior to exposure to the aorta. Adhesion of B16F10 to the aorta was significantly reduced in the presence of RGDS in intact or vessels denuded of endothelium compared to adhesion in the absence of RGDS (**denotes statistical significance of RGDS and RGDS plus morphine treated groups in intact or denuded of aorta to other groups; *denotes statistical significance of the untreated TC on EC-intact aorta to other groups and *$P < 0.01$, Wilcoxon signed ranks test). Exposure of TC to RGDS prior to their addition to control or morphine treated aorta resulted in adhesion that was significantly less than that seen following the addition of untreated TC to control aorta. When TC were pretreated with RGDS peptide, adhesion was similar, regardless of whether aorta were intact or denuded, or treated with morphine or vehicle only. The data shown are the median of N=3 experiments.

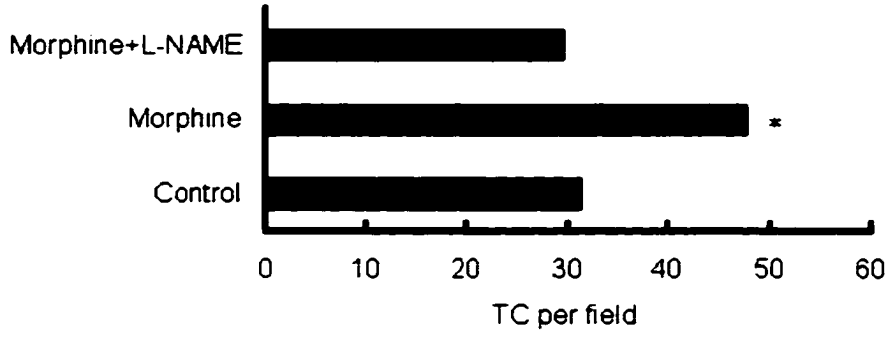


Figure 4.8. Increased adhesion to aorta exposed to morphine *in vitro*.

The experiments were performed as described in Figure 4.3 except that morphine was injected IP into C57BL/6 mice 1 hour prior to sacrifice and removal of the aorta. *In vitro* adhesion of B16F10 to the aorta 1-2 hours after treatment with morphine was significantly increased relative to untreated controls ($P < 0.005$, Wilcoxon signed ranks and ANOVA). Injection of mice IP with L-NAME 30 min prior to injection with morphine abrogated the increased adhesion of B16F10 to aortic segments *in vitro*. The data shown are medians of N=5 experiments).

CHAPTER FIVE

MORPHINE REDUCES ADHESION OF ENDOTHELIUM-BOUND B16F10 MELANOMA ON MOUSE AORTA 48 HOURS AFTER DRUG TREATMENT AND THIS AFFECT IS ECNOS AND NO DEPENDENT

Introduction

In the previous chapter, we demonstrated that morphine augments TC adhesion to the mouse aorta 1 hour after drug treatment, and that this increase required the presence of the EC layer and NO synthesized by EC-NOS. We also demonstrated that a single dose of morphine administered as an intraperitoneal inoculation caused an EC-NOS and NO dependent increased in adhesion of TC to the endothelium of aortic ring segments 1-2 hours after drug treatment. Recently, Dr. Szalay *et al* (manuscript in preparation) demonstrated that a single dose of morphine administered intraperitoneally to mice prior to removal of a tumor-filled eye was capable of significantly decreasing spontaneous pulmonary metastasis. Using cell tracker labeled TC and quantitative confocal microscopy of whole lung mounts they found an

increased retention of melanoma cells in the small pulmonary vessels of the lung 1-2 hours after intraperitoneal administration of a single dose of morphine to the mice. Interestingly, these results are consistent with a hypothesis of increased adhesion of TC to vascular EC at this early time interval, and with the in vitro findings described in the present thesis. Additional experiments in Dr. Szalay's laboratory demonstrated that the initial increase in retention of viable TC in the lung was followed by a decreased retention of TC observed at 48 hrs. No differences were observed in intraluminal TC aggregation, or TC-platelet aggregation at either 2 or 48 hours. If the decreased retention of TC observed in vivo at 48 hrs does indeed reflect decreased adhesion of TC to pulmonary vessels, we would predict a decrease in adhesion of TC to aortic strips 48hrs after treatment of aortic rings with morphine.

In order to evaluate the fate of the adherent TC on aorta, we therefore quantitated the adherent TC in 2 and 48 hours after TC were placed luminal surface of morphine-treated aorta. The results indicated morphine induces and increase in adhesion of TC to the aortic rings at 2 hours, but reduces the numbers of adherent TC at 48 hours. This decreased adhesion required the presence of EC NOS, and NO.

Method and material

Animals

C57BL/6 mice (Charles River, Ballardville Mass), and C57BL/6.129P2-NOS3^{tm1Unc} mice (Jackson Laboratories, Jacksonville, Florida) were 8 – 12 weeks of age when used in experiments.

Melanoma cell preparation

B16F10 cells were harvested using trypsin, 0.25% and incubated with 10 μM Cell Tracker (Molecular Probe) for 40 min at 37°C to fluorescently-label the cells. Cell Tracker labels only live cells and did not alter B16F10 vascular adhesion or metastasis. Unincorporated dye was removed by extensive washing with DMEM whereupon the cells were resuspended in fresh DMEM and maintained at 37°C prior to use in assays.

Melanoma adhesion to the vascular endothelium

Aortic segments were excised from mice and prepared for use exactly as described above for NO determination except that prepared tissue was placed in DMEM and maintained at 37°C, 5 % CO_2 for the duration of the assay. The aortic ring was cut, exposing the luminal surface and a notch was created in the upper left corner to facilitate orientation of the tissue upon conclusion of the assay. The aorta was treated with buffer or 1 μM morphine for 20 minutes prior to addition of the cell-tracker-labeled B16F10 cells ($5x10^4$

cells/ml) for 2 or 48 hours. Aortic segments were rinsed with pre-warmed PSS to remove non-adherent cells whereupon the tissue was fixed with 3.7% formaldehyde for 15 minutes at room temperature, placed on a slide with the lumenal surface up and mounted with a coverslip using gel-mount media. B16F10 adhesion to the lumenal surface of the aorta was evaluated using quantitative fluorescent confocal microscopy and a Meridian Ultima confocal microscope. Tissue was visualized using a 40x oil immersion lens and 5 random fields of view were evaluated per aorta. For quantitation of B16F10 adhesion, the PMT detector was set a level where only the cell-tracker-labeled B16F10 cells were visualized and autofluorescense of the tissue was negated. The pinhole setting on the confocal microscope was set to 100 μm so that only the lumenal surface was visualized. For experiments that evaluated *in vivo* exposure to morphine, mice were injected IP with 5 mg/kg morphine, saline or L-NAME only, or L-NAME followed 30 minutes later by morphine whereupon the mice were sacrificed and the tissue was processed exactly as described above for *in vitro* studies.

To evaluate the direct effects of NO on TC adhesion, experiments were performed exactly as described above except that aorta was treated with the NOS inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME), 100μM for 20 min and washed extensively prior to exposure to TC. To evaluate the effects of the endothelium on TC adhesion, endothelium cells were removed by

scraping prior to exposure to vascular tissue and the experiments were completed exactly as described above.

Results

Morphine decreases TC adhesion 48 hours after drug treatment of aortic rings.

Adhesion of B16F10 cells to the lumenal surface of mouse aorta was evaluated in the presence or absence of morphine at 2-or 48-hour after cell addition to the aortic rings. Adhesion of B16F10 cells was increased markedly 2- hour after exposure of aorta to morphine. This observation is similar to our previous findings in which morphine induced TC adhesion 1-hour after addition of TC to aorta (Figure 5.1). These results indicate that TC adhesion to the aorta significantly decreased between 2- to 48-hours incubation. Furthermore, after the 48-hour incubation, fewer adherent TC were detected in morphine–pretreated aorta tissue than in the placebo (Figure 5.1).

The morphine-facilitated TC clearance from the aortic required the presence of EC

In order to evaluate the role of endothelium in morphine-facilitated reduction of TC adhesion after a 48-hour incubation, B16F10 cells were placed on either endothelium-intact or denuded aorta. The results showed that in the endothelium-intact aorta treated with morphine, fewer adherent cells were present, however, in the denuded aorta morphine had little or no significant impact on TC adhesion (Figure 5.2). Furthermore, B16F10

retention on aorta tissue is remarkably increased in denuded mouse aorta either with or without the presence of morphine.

NOS3 is involved in the reduction of B16F10 adhesion 48 hours after treatment of aortic rings with morphine.

It has been reported that NO is mediated cytotoxicity of B16 melanoma in hepatic sinusoidal endothelium *in vivo* (Carretero *et al.*, 2001; Wang *et al.*, 2001). Therefore, it suggests that our observation of morphine-induced reduction of TC adhesion on aorta *in vitro* may be a late response mediated by NO release from endothelium. In order to determine the role of NO and NOS, we tested the effect of morphine on the adhesion of B16F10 cells to the aorta of NOS3 knockout mice. In NOS3 knockout mice, morphine failed to increase TC adhesion after 2-hour or decrease TC adhesion after 48-hour. This demonstrates that the mechanism of morphine-induced reduction of TC adhesion to the aorta at 48 hours *in vitro* requires the presence of endothelium, and of NOS3.

Discussion

In this chapter, we have demonstrated that morphine can reduce endothelium-bound TC adhesion to the aorta 48 hours after drug treatment. However, this mechanism is abolished in the denuded aorta and in NOS3 knockout mice, suggesting the requirement of EC-NOS. These results also suggest that NO may be involved in this process of TC clearance from the aortic endothelium. However, either the dissociation of viable TC from the endothelium, or TC lysis induced by morphine may contribute to the reduction at 48 hrs of TC adhesion *in vitro*. The reduced adhesion seen in vitro at 48 is consistent with a hypothesis that the decreased retention of TC observed *in vivo* in pulmonary vessels after 48 hrs may reflect a decreased adhesion of TC to the endothelium (Adjodha *et al.*, 2001).

It has been reported that exogenous NO released by SNAP is able to induce apoptosis in various cell types including TC cell lines *in vitro*. Therefore, NO induced by the aorta endothelium *in vitro* may promote bound TC to undergo apoptosis. In fact, it has been reported that the NO-induced TC cytotoxicity alone with extravascular oxidative stress elicited by leukocytes and endothelial cells contribute to the elimination of circulating and capillary-arrested cancer cells *in vitro* (Glaves, 1986; Weiss *et al.*, 1988 Carretero *et al.*, 2000; Wong *et al.*, 2000).

The biological effects of nitric oxide have been extensively studied and This molecule has been suggested to play a central role in the tumor cell arrest on capillaries and metastasis by regulating vasodilatation, platelet aggregation, angiogenesis, production of prostaglandins, or leukocyte proliferation, or mediating tumor cytotoxicity (Wink *et al.*, 1998). NO-mediated tumor cytotoxicity may result from its diverse biological functions. NO reversibly binds to cytochrome c oxidase and may inhibit respiration. NO affects the activity of membrane bound, cytosolic and nuclear proteins as well as transcription factors such as nuclear factor -κB and Sox R, so that NO may affect tumor cell growth and development. Furthermore, NO plays a significant role in regulating apoptosis and necrosis of tumor cells. It promotes S-nitrosylation of caspases and tissue tranglutaminase, which are associated with apoptosis and necrosis, resulting in modifying their activities. (Melino *et al.*, 1997). Moreover, the free radical, $NO^{\cdot}$ and –OONO derived from nitric oxide may also directly contribute to cytotoxicity on tumor cells. In recent studies, it has been demonstrated that the hepatic sinusoidal endothelium can deliver cytotoxic effects on endothelium-bound tumor (Carretero *et al.*, 2001; Wang *et al.*, 2001). This discovery suggests that NO released from sinusoidal endothelium induces endothelium-bound TC apoptosis. This discovery suggests that the tumor cytotoxicity of hepatic sinusoidal endothelium via NO release is a natural self defense mechanism

against metastasis. This mechanism alone, or with host immune function may contribute to the causes of low successful metastasis rate of circulating tumor cells to form a secondary tumor in a distant organ. (Fidler, 1970).

In our system, the mechanism responsible for the decreased adhesion of TC to the endothelium remains unclear. In previous studies on the activities of human monocytes modulated by the NO donor, SNAP, we found (Magazine *et al.*, 2000) NO initially inhibits monocytes in several aspects including cell activation, phagocytosis, chemotaxis and cytoskeletal actin polymerization and this inhibition lasts for at least 2 hours. Therefore, we consider the possibility that after 2 hours the increased expression of adhesion molecules induced by morphine on the surface of TC or EC become internalized or down-regulated, resulting in a decrease of viable TC from the vascular endothelium. Alternatively, it is possible that a delayed signal transduction pathway involving a morphine-induced and NO mediated TC killing may become activated. This would may be consistent with the hypothesis that the endothelium *in vivo* may serve as a cell trapper to recruit free circulating TC on vascular endothelium and eventually destroy them by an NO-mediated mechanism. Therefore, administration of NOS3 stimulating drugs or cytokines may yield beneficial results in treating cancer by facilitating destruction of vascular endothelium-bound TC in *vivo*.

In summary, our data suggests that morphine regulates TC adhesion to EC *in vitro* by two stages: at first, morphine-induced NO promotes TC arrest on vascular bed; then by 48 hours more delayed reactions resulting from NOS3 stimulation and NO release-mediate either the destruction of endothelium-arrested TC, or their release from the endothelium.

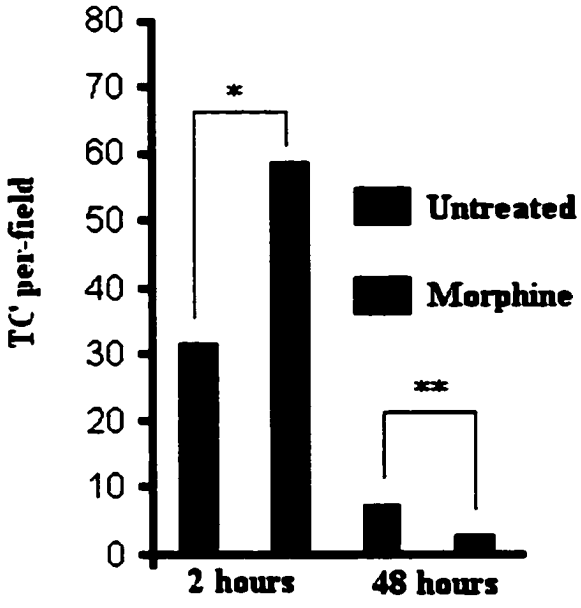


Figure 5.1. A transient morphine-induced enhancement of B16F10 adhesion to aortic rings exposed to morphine is diminished after 48 hours.

This experiment was performed as described in Chapter 4. Cell adhesion to the mouse aorta was evaluated at t 2 (short term) and 48 hours (long term) after cells were placed on aortic rings. As we previously described, B16F10 adhesion was increased in morphine-pretreated mouse aortic rings after 2 hours, while at 48 hours fewer B16F10 cells were present than in the control. The data shown are the median of N=4 experiments, *$P < 0.01$ denotes statistical significance between morphine and untreated; ** $P < 0.05$ denotes statistical significance between morphine and untreated rings; the statistical significance was analyzed by the Wilcoxon signed ranks test).

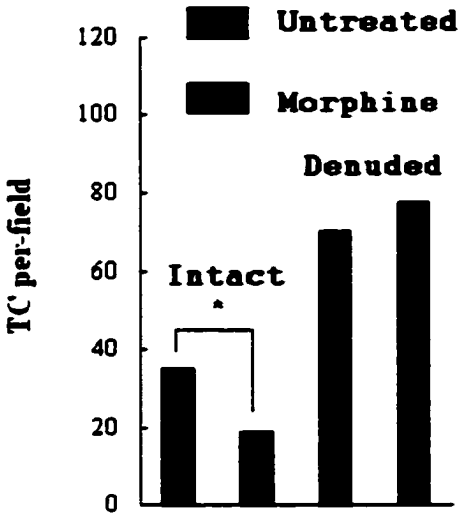


Figure 5.2. The morphine-induced decrease in adhesion of B16F10 cells to the aorta at 48 hrs requires the presence of the endothelium.

Experiments were performed as described in Figure 3 of the previous chapter except that some aortic rings were denuded of endothelium. Adherent cells were measured 48 hours after addition to aortic rings. In the endothelium-intact aorta, significantly fewer B16F10 cells were present on the morphine–pretreated intact aorta than on the untreated aorta. However, in denuded aortic rings, no significant difference was seen between the morphine treated and control aorta. The data shown are the medians of N=4 experiments, *P < 0.01:denotes statistical significance between adhesion to the morphine treated and untreated-EC intact aorta. Statistical significance was analyzed by the Wilcoxon signed ranks test).

Figure 5.3. B16F10 adhesion on NOS3 knockout mouse aorta following morphine exposure.

Experiments were performed as described in Figure 3 of chapter 4 except that the aorta was from the NOS3 knockout mouse (C57BL/6.129P2-NOS3^{tm1Unc}). Morphine fails either to increase cell adhesion in 2 hours or to decrease cell adhesion after 48 hours incubation with NOS3 knockout mouse aortic rings. (The data shown are the medians of N=4 experiments).

CONCLUSION

In chapter one, we demonstrated that morphine induced NO production from THP-1 but failed to induce detectable amounts of NO from B16F10 cells. In chapter 2, we found that stimulation of morphine opioid receptors induced an NO-dependent cell rounding in THP-1, but failed to induce a conformational change in B16F10 cells. In chapter 3 we demonstrated that a morphine-mediated NO release induced cytoskeletal actin depolymerization in THP-1 but not in B16F10. It has been reported that NO induces F-actin disassembly (Sandau *et al.*, 2001) and our observation is consistent with those studies. Furthermore, our studies demonstrate that a morphine-induced NO release prompts re-arrangement of the cytoskeletal actin network, resulting in a cellular morphology change from amoeboid shape to round in THP-1.

Beside the effect of morphine on inducing morphological changes and rearrangement of the actin cytoskeleton in THP-1 cells, we demonstrated that morphine inhibits proliferation and viability of both THP-1 and B16F10 cell lines, and demonstrate that the cytotoxic effect(s) induced by morphine in both cell lines does not appear to be mediated by apoptosis. This suggests that morphine-mediated cytotoxicity in these TC is likely associated with a cellular necrotic mechanism.

Both cancer cell lines showed a differential susceptibility to morphine, and B16F10 cells are more sensitive than THP-1 cells. Since B16F10 cells

fail to release NO in response to morphine, it seems that NO is not likely to play a role in the morphine-mediated adverse effect on proliferation or viability in B16F10. This suggests that the adverse affect on B16F10 cells may occur in an NO-independent manner. This hypothesis receives further support from our invasion studies that demonstrate an action of morphine on B16F10 cells, independent of NO (see below). In comparison with B16F10, THP-1 is more resistant to morphine. A low dose of morphine (1 μM) did not induce significantly the adverse effects in THP-1, while with a higher dose did (100 μM) significantly affect proliferation and viability. We don't have sufficient data to determine whether the morphine-mediated adverse effect on THP-1 cell viability is NO-dependent or NO-independent since neither the NOS inhibitor (L-NAME) nor the potent NO scavenger, (carboxyl-PTIO) were used in this experiment. However, our invasion studies suggest that morphine can affect THP-1 cells in both an NO-dependent, and an NO-independent manner, and need not involve opioid receptors.

Examination of invasion, demonstrates that morphine inhibits invasion of both THP-1 and B16F10 cells through a fibronectin barrier in Boyden chambers. In THP-1 cells this morphine-induced inhibition is only partially dependent on NO production and NOS activation and appears to be affected by activation of a non-opioid receptor-mediated pathway. In contrast, in B16F10 cells the inhibitory action of morphine is completely independent of

NO production, but completely dependent upon stimulation of opioid receptors. Taken together the invasion data suggests that morphine is capable of inhibiting invasion, and may act independently of opioid receptors, or through an opioid receptor mechanism that may or may not be NO-dependent. The mechanism of morphine-induced inhibition of TC invasion *in vitro* has not been elucidated in the present studies. However, we do show that morphine fails to elicit NO production, cell rounding or visible alteration of the cytoskeleton in B16F10 cells. Thus morphine's effect on invasion in this melanoma cell probably does not involve alterations of the actin cytoskeleton or cell motility. We speculate that morphine may affect invasion by altering protease secretion. According to published literature, morphine or morphine-mediated NO may exert an inhibitory effect on TC invasion by inhibiting matrix metalloproteinase expression/activity, resulting in an inhibition of TC dissemination and extravasation. It has been reported that morphine inhibits indolactam V-induced U937 cell gelatinase (a subgroup of matrix metalloproteinase, MMP that degrade type IV collagen) secretion (Week *et al.*, 2001) and modulates 72- KD MMP activity in mesangial cells (Sagar *et al.*, 1994). This suggests that morphine may inhibit MMP activity and secretion from the THP-1 and B16F10 cell lines and prevent degradation of ECM proteins. It also has been reported that ecNOS gene transfer inhibits smooth muscle migration and MMP-2 and MMP-9

activity (Milind *et al.*, 1999), suggesting NO decreases MMP-2 and MMP-9 activities and increases TIMP-2 (tissue inhibitor of MMP) secretion, and this shifts the balance of MMP activity, which may favor the inhibition of cell migration because of inhibition of extracellular matrix degradation. Taken together, the above information implies that morphine alone or morphine-mediated NO may modulate MMP activity of THP-1 and/or B16F10 cells, resulting in an inhibition of ECM degradation and TC invasion.

In chapter 4, we demonstrated that morphine increased B16F10 adhesion to the mouse aortic endothelium 1-2 hours after drug treatment and that this action was EC-NOS3 and NO dependent. Similar results were observed after treatment of mice with a single intraperitoneal analgesic dose of morphine. We also demonstrated that the altered adhesion of melanoma cells to the endothelium observed at 1-2 hours likely involved an NO dependent alteration of expression of cognate receptors for melanoma cell integrins on the EC. This morphine-induced increase in adhesion to EC-receptors suggests that the affects of morphine on adhesion may be highly dependent on cell type.

In Chapter 5 we demonstrated that 48 hours after the aortic endothelium is exposed to morphine, adhesion of B16F10 cells is dramatically decreased, and the decrease is endothelium, as well as NOS3-, and NO- dependent. These results show that long term responses initiated by NO release impact

the release of either viable or dead cells from the aortic endothelium. However, preliminary examination of cell viability after 48 hours of incubation of TC with aortic rings demonstrates no adverse drug affects on melanoma cell viability (data not shown).

Collectively, our data shows that exposure of vascular preparations to morphine can alter the adhesion of TC to the vascular endothelium in an EC-NOS3 and NO-dependent manner. These affects may be dependent on cell type, and on NO-induced time-dependent changes occurring in the EC, and/or TC.

At present, most studies involving TC and morphine acknowledge the well document ability of morphine to induce immunosuppression in cancer patients, Importantly, however, the above studies on proliferation, viability, invasion, and adhesion, suggest varied ways that morphine could act in vivo to modulate TC metastasis. However, there are a lot of remaining questions to be answered. In order to resolve these questions, further studies need to be proposed. In my future studies, I would like to evaluate the effects of morphine and morphine-mediated NO on modulation of TC MMP and TIMP secretion and relate this to invasion *in vitro*. Second, I would like to evaluate expression of adhesion molecules and receptors such as: ICAM, selectins and integrins, on endothelium and tumor cells following exposure to morphine in a short and long term basis. These studies may help us to understand the

mechanism of morphine-induced alterations of short-term and longer-term adhesion. Third, I would like to characterize the mechanism of morphine-induced TC clearance from the aortic endothelium after a 48-hour exposure to drug. This mechanism is likely associated with either an EC-mediated and NO-induced apoptosis, or with a TC release from EC resulting from a morphine-induced down-regulation of adhesion molecules, receptors and integrins. Finally, I would like to use the intravital microscopic technique to evaluate TC adhesion in the microcirculation *in vivo* following I.V. injection of morphine and other NO-generating drugs. This semi-*in vivo* technique may allow us to observe the interaction between TC and EC of cremasteric microcirculation in a dynamic manner, and help us to understand the morphine-modulated TC adhesion to endothelial cells *in vivo*. Further characterization of the mechanism(s) of action, and the complex effects of morphine on TC in general, and melanoma cells in specific, should yield important information that could be utilized in experimental strategies designed to decrease metastasis.

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