Nitric Oxide/Peroxynitrite Imbalance Induces Adhesion of Cancer Cells to Lymphatic Endothelium - Clinical Implications for Cancer Metastasis

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Yuanyuan Tang
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This dissertation titled
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by

YUANYUAN TANG

has been approved for
the Department of Chemistry and Biochemistry
and the College of Arts and Sciences by

Tadeusz Malinski
Professor of Chemistry and Biochemistry

Robert Frank
Dean, College of Arts and Sciences
Abstract

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Nitric Oxide/Peroxynitrite Imbalance Induces Adhesion of Cancer Cells to Lymphatic Endothelium - Clinical Implications for Cancer Metastasis

Director of Dissertation: Tadeusz Malinski

Adhesion of cancer cells to endothelial cells is a key step in metastasis. However, the molecular mechanisms mediating cancer cell/endothelial cell interaction and adhesion process are not fully understood. Nitric oxide (NO) has been found to play an important role in the regulation of local blood flow and may be involved in lymph node metastasis. It has also been shown that the effects of NO are difficult to separate from the effects of its byproduct, peroxynitrite (ONOO$^-$). In this study, we used a nanomedical approach to examine the imbalance between cytoprotective NO and cytotoxic ONOO$^-$ in lymphatic endothelial cells, a key mediator of cancer cell adhesion. By using electrochemical nanosensors, we monitored in vitro NO and ONOO$^-$ release from human lymphatic endothelial cells (HLECs) stimulated by cancer cells. Our study showed that at low [NO]/[ONOO$^-$] ratio (lower than 1.5), the adhesion of CACO-2 or HT1080 cancer cells to HLECs increased. An increase level of [NO]/[ONOO$^-$] with agents that facilitate NO formation (e.g., SNAP, PEG-SOD, MnTBAP, VAS2870 and L-arginine) suppressed lymphatic metastasis of CACO-2 human colon cancer cell line and the highly invasive HT1080 human fibrosarcoma cell line through inhibition of the surface expression of intercellular adhesion molecule 1(ICAM-1) and vascular cell adhesion molecule 1(VCAM-1) on HLECs. Collectively, this study demonstrates for first time the crucial
role of the [NO]/[ONOO⁻] balance in cancer cell-lymphatic endothelial cell interaction, which may be exploited clinically to prevent lymphatic metastasis.
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List of Abbreviations

**BH4** tetrahydrobiopterin

**CLEVER-1** common lymphatic and vascular endothelial receptor

**CTCs** circulating tumor cells

**eNOS** constitutive nitric oxide synthases

**DPBS** Dulbecco's Phosphate-Buffered Saline

**eNOS** endothelial nitric oxide synthases

**EDHF** endothelium-derived hyperpolarizing factor

**EMEM** Eagle's Minimum Essential Medium

**ESR** electron spin resonance

**FAD** flavin adenine dinucleotide

**FMN** flavin mono nucleotide

**H4B** (6R)-5,6,7,8-tetrahydro-L-biopterin

**HLEC** human lymphatic endothelial cells

**HUVEC** human umbilical vascular endothelial cells

**ICAM-1** intercellular adhesion molecule-1

**INCAM-110** Inducible cell adhesion molecule 110

**IL1-β** interleukin-1β

**iNOS** inducible nitric oxide synthases

**L-NAME** Nω-nitro-L-arginine methyl ester

**LYVE-1** lymphatic vessel endothelial HA receptor-1

**MCP-1** monocyte chemoattractant protein-1
MnTBAP Mn(III)tetrakis (4-benzoic acid) porphyrin chloride
MR mannose receptor
NAD(P)H reduced nicotinamide adenine dinucleotide
NF-κB nuclear factor κB
Ni-TMHPP Ni-tetrakis(3-methoxy-4-hydroxyphenyl) porphyrin
nNOS neuronal nitric oxide synthases
PBST Phosphate-Buffered Saline with Tween 20
PEG-SOD polyethylene glycol-superoxide dismutase
ROS reactive oxygen species
SIN-1 3-morpholine-syndnonimine
sLea sialyl-Lewis a
sLex sialylated-Lewis x
SNAP S-nitroso-N-penicillamine
TMHPP tetrakis(3-methoxy-4-hydroxy-phenyl)porphyrin
TNF-α tumor necrosis factor-α
VCAM-1 vascular cell adhesion molecule-1
VEGFR-3 vascular endothelial growth factor receptor-3
VEGF-C vascular endothelial growth factor-C
VEGF-D vascular endothelial growth factor-D
1. Introduction

Metastasis is a complex process in which cancerous cells detach from the primary tumor, invade the extracellular matrix and enter the circulation system. When cancer cells break away from a tumor, they travel to other areas of the body through the bloodstream or the lymph system. Adhesion of circulating cancer cells to endothelium, which lines the inner surface of blood vessels and lymphatic vessels, is the first step of tumor cell invasion into distant organs (1). Previous research has shown that recurrence and metastasis of cancer are closely related to the adhesion between tumor cells and endothelial cells. The adhesive interactions of circulating tumor cells with endothelial cells facilitate their survival and extravasation from the vasculature (2). Understanding the molecular mechanisms of adhesive interactions between tumor cells and endothelial cells may provide guidelines for developing promising anti-metastatic therapies.

The lymphatic system is the primary route for many types of metastatic cancer, which is the major cause of death in cancer patients. It has been estimated that 80% of metastasis of solid cancers disseminate through the lymphatic system, while 20% of metastases may occur through the blood vasculature or by direct seeding (3). While, most of the current research has focused on the molecular mechanism of cancer cell adhesion to blood endothelial cells, little is known about cancer cell attachment to lymphatic endothelial cells and its relationship to lymph node metastasis (2,3). A better understanding of the underlying mechanisms is crucial for effective treatment for metastatic cancer. The current study focuses on the adhesion of tumor cells to lymphatic endothelium.
1.1. The lymphatic system

1.1.1. The lymphatic system

The lymphatic vascular system is a complex network comprising blind-ended capillaries, collecting vessels, lymph nodes, lymphoid organs and circulating lymphocytes. Lymphatic vessels are distributed to most organs, except the central nervous system, bone marrow, cartilage, cornea and epidermis (5). A number of important physiological functions have been described. It maintains tissue fluid balance by absorbing and draining fluid and macromolecules extravasated from blood vessels and returning them back into the blood circulation. Lymphatic vessels can also absorb fat from the gut (6). Furthermore, the lymphatic system can direct leukocytes and antigens to lymph nodes thereby playing an essential role in the body’s immunological surveillance system (7).

Due to their dual roles of fluid absorption and lymph transport, the structures of lymphatic vessels differ from blood vessels. Lymph capillaries are lined by a layer of endothelial cells supported by a discontinuous basement membrane (8). The lack of tight connections between endothelial cells allows other cells, whether they be immune-related or malignant, to enter and exit the lymphatic vessels (9). In addition, the wall of lymphatic endothelial cells is joined to the extracellular matrix by anchoring filaments, enabling lymphatic vessels to open and function. Furthermore, chemotactic agents secreted by LECs can attract tumors cells toward lymphatics (5). A good example is CCL21 which interacts with CCR7 on some tumor cells (5).
1.1.2. The vascular and lymphatic endothelium

The blood vasculature and the lymphatic vasculature are the two systems involved for effective circulation. The blood vasculature delivers oxygen and nutrients and removes waste products for detoxification and replenishment, while the lymphatic vasculature regulates the fluid balance in tissues by returning excessive fluid from the interstitial space back to the bloodstream (7). The endothelium lines the interior surface of blood vessels and lymphatic vessels and regulates a variety of physiological processes such as angiogenesis, vasoregulation and metastasis (10). This last function is particularly important because endothelial cells constitute the interface between circulating cancer cells and possible sites of metastasis (11). The lymphatic endothelium and vascular endothelium have many common properties. Some adhesion receptors representative of all major adhesion receptor families such as CD31 and VE-cadherin are expressed on both cell types (12).

The lymphatic endothelium and vascular endothelium also have distinct characteristics and functions (13). For example, Interleukin (IL)-8, IL-6, the chemokine receptor CXCR4, ICAM-1, Integrin α5 are expressed in higher levels in vascular endothelial cells (VECs) (2). Some specific markers for the lymphatic endothelium were also identified, including vascular endothelial growth factor receptor-3 (VEGFR-3), lymphatic vessel endothelial HA receptor-1 (LYVE-1), Podoplanin, Prox-1, and D2-40 (14).
1.1.3. The lymphatic system in cancer

The most common pathway of initial metastasis is through the lymphatic system. In fact, lymph nodes are the most common sites of metastasis for many cancers (3). There are likely to be several reasons for this: specific structures of lymphatic vessels, the lymph flow, chemokines, cell adhesion molecules and lymphangiogenesis (7).

Lymphatic vessels are known as primary routes of tumor dissemination (15). Unlike blood vessels, lymphatics have looser junctions between the ECs. Additionally, their basement membrane is discontinuous and are not surrounded by pericytes, thus making them relatively permeable to invading tumor cells (7). Furthermore, the wall of lymphatic endothelial cells are connected to the extracellular matrix by anchoring filaments, enabling lymphatic vessels to open and function (16). Tumor cells can take advantage of these structural characteristics to enter the lymphatic system (5). In addition, metastatic cells are filtered into the lymph sinus upon reaching the node and may encourage the formation of tumor cell aggregates that could protect against tumor cell death (17). Furthermore, the low-pressure lymph flow also likely favors the survival of circulating tumor cells, while tumor cells in the bloodstream experience high shear stresses and mechanical deformation leading to an extremely low success rate for metastasis (18).

Tumor cell attachment to lymphatic endothelium may also be mediated by cell–cell adhesion molecules expressed on the surface of lymphatic endothelium. The LEC-specific mannose receptor (MR) and the common lymphatic and vascular endothelial receptor (CLEVER-1) are the first molecules identified to be involved in adhesion of
head and neck squamous cell carcinoma to lymphatic endothelium (19). Additionally, MR expression on lymphatics within breast carcinomas was associated with cervical lymph node metastases (9). Discovery of specific molecules involved in tumor cell-lymphatic endothelial cell interactions could provide new insights for treating metastasis (9).

The effect of chemokines on interactions between lymphatic endothelial cells and tumor cells are areas of intense interest. Recent findings indicate that chemokines produced by lymphatic endothelium lead cancer cells into the lymphatic vessels, while neutralizing antibodies to these chemokines or their receptors have been shown to reduce risk of developing metastatic cancer (3). Initial studies suggested that CCL21 and CCR7 play an important role in lymphatic metastasis. High expression of CCR7 in B-16 murine melanoma cells leads to chemoattraction through binding with CCL21 on LECs. Neutralizing antibodies to CCL21 significantly reduced risk of metastasis (20). The CXCL12-CXCR4 pair has also been found to be involved in lymphatic metastasis. CXCL12 overexpression in lymphatic vessels has been shown to facilitate lymphatic metastasis in a mouse model, which was completely blocked by antibodies to CXCR4 receptors on malignant cells (3). Another chemokine receptor important for metastasis is CCR8. Activation of tumor cell CCR8 by CCL1 leads tumor cells into lymphatic vessels and allows subsequent formation of lymph node metastases (21). Lymph node metastasis was significantly reduced by blocking CCR8 with antagonist and short hairpin RNA in a mouse model (21).
Solid tumors require growth of new vessels to grow in size. Lymphangiogenesis is the growth of new lymphatic vessels and is activated in cancer and inflammation (3). Lymphangiogenic growth factors such as vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor-D (VEGF-D) can enhance tumor lymphangiogenesis and lymphatic metastasis (15). Zeng and coworkers examined the expression of VEGF-C, VEGF-D, and VEGF receptor-3 in prostate cancer specimens and suggest that VEGF-C and VEGF-D may be involved in lymphatic metastasis through activation of lymphatic endothelial cell VEGF receptor-3 (22). Another study showed that transgenic mice that overexpressed VEGF-C had a higher rate of lymph node metastases than did wild-type mice (8). Together, these results provide strong support for the contribution of VEGF-C, VEGF-D, and their receptor, VEGFR-3, in lymphatic spread in malignancy and the ability to inhibit lymphangiogenesis and lymphatic metastases with antibodies against VEGF-C, VEGF-D, or VEGFR-3 suggests potential therapeutic approaches (7). In fact, over 70 angiogenesis inhibitors have been developed to block tumor angiogenesis and have shown promising preliminary results (12). Since specific growth factor receptors are selectively expressed on angiogenic vessels compared to the normal vasculature, these treatments would have minimal adverse effects on normal tissue (12).

1.1.4. The lymphatic endothelium-cancer cell interaction

Whereas mechanisms involving blood vessels in tumor growth and metastasis have been studied extensively over the past few years, little effort has been directed toward understanding the mechanisms of cancer cell and lymphatic endothelium
interaction and its relation in tumor progression and lymphatic metastasis. Interactions between disseminated tumor cells and endothelial cells are critical for metastatic colonization. Therefore there is currently a great need for clarifying the interactions between tumor cells and lymphatic endothelium and to develop a paradigm for lymphatic metastasis similar to that of hematogenous metastasis.

Bevacqua and coworkers studied the interaction of several human tumor cell lines with bovine lymphatic endothelium and showed that tumor cell lines with fibroblastic-like morphology displayed more rapid adhesion than tumor cell lines with more rounded morphologies (23). Another study examined the adhesion pattern of BV9, a rat gastric adenocarcinoma cell line, to lymphatic endothelial cells and found that high affinity between cancer cells and LEC was associated with high possibility of lymph node metastasis, which can be augmented by inflammatory stimulus such as IL1-β or TNF-α (24). Similarly, TNF-α stimulation of both human dermal lymphatic endothelial cells and tumor cells (e.g., MCF-7 breast cancer cells, SKMEL-30 melanoma cells) was found to significantly increase tumor-endothelial cell adhesion under static conditions, while TNF-α stimulation of endothelial cells, or tumor cells alone, did not change the adhesion patterns, suggesting that cytokines may regulate lymphatic metastasis resultant from tumor cell-lymphatic endothelial cell adhesion (25). In addition, cancer cell lines expressing CCR7 have been shown to have increased affinity for lymphatic endothelium and increased lymph node metastasis in vivo, which can be inhibited by neutralizing anti-CCL21 antibodies (26). Inhibition of cell-cell interactions may be a useful target for reducing risk of metastasis (27).
1.2. Nitric oxide

1.2.1. The role of nitric oxide in biological system

Nitric oxide (NO) is synthesized from oxygen and L-arginine by nitric oxide synthase (NOS) and has been identified as an endothelium-derived relaxing factor which participates in many biological processes including the regulation of vascular tone, inflammation, and apoptosis (28). Besides being the most potent endogenous vasodilator, NO also inhibits smooth muscle cell proliferation and migration, adhesion of leukocytes to the endothelium, and platelet aggregation (28). A variety of stimuli, including increased vascular flow, and pharmacological agents, such as acetylcholine, bradykinin, ATP, and histamine, produce vascular relaxation by the release of NO (29).

Three isoforms of nitric oxide synthase have been identified: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS isoform (iNOS) (30). nNOS and eNOS are constitutively expressed by neuronal and endothelial cells, whereas iNOS is induced by inflammatory cytokines, endotoxin, hypoxia, and oxidative stress (31). All three isoenzymes are homo-dimers and each monomer contains four prosthetic groups: FAD (flavine adenine dinucleotide), FMN (flavine mononucleotide), H4B ((6R)-5,6,7,8-tetrahydro-L-biopterin) and protoporphyrin IX heme (32).

eNOS is the primary isoform responsible for the production of NO within blood vessels (33). NO synthesis involves electron transfer between different cofactors including FAD, FMN and NADPH (nicotinamide adenine dinucleotide phosphate), H4B, and calmodulin. The concentration of L-arginine and H4B is mainly responsible for NO
production. L-Arginine analogues such as D-arginine and N-methylester (L-NAME) are not suitable as NOS substrates and can competitively inhibit NO formation (32).

NO is lipophilic; therefore it can freely diffuse through cellular membranes. NO is a free radical with a short lifespan (half-life 2–5s) and may react with a few types of molecules inside or outside the cell. NO can react with \( O_2 \) to form nitrogen dioxide (\( NO_2^- \)), or superoxide to produce peroxynitrite (\( ONOO^- \)). NO may also react with a few metal ions such as iron, copper or manganese, which are usually bound to proteins. Most of these products are more reactive than NO. For example, \( ONOO^- \) is much more cytotoxic than NO and can cause DNA damage. Thus, the reactivity of NO dominates the chemistry of NO in the biological systems.

1.2.2. Detection of nitric oxide

Monitoring NO in biological systems is very challenging since NO is highly reactive in aqueous systems. It can react rapidly with \( O_2^- \), \( O_2 \) and a few metal ions within proteins such as iron, copper and manganese. This leads to a short half-life (1-6s) and low concentrations (range from picomolar to micromolar) (34). Despite such challenges, many techniques have been developed for NO detection. Indirect methods include chemiluminescence, UV-visible spectroscopy and electron spin resonance (ESR) spectroscopy which rely on measurements of secondary species. Electrochemical detection is the only method for monitoring NO directly \( in vivo \) and \( in vitro \) (35). This method is based on the oxidation of NO on chemically modified electrodes. Therefore, most NO sensors are based on the oxidation of NO. The design of organized layers on
electrode surfaces can improve the electrode selectivity for NO. The electrode can be coated with either a membrane, which serves as a filter for interferents such as nitrite (NO$_2^-$), or an electrocatalytic compound, which lowers the NO oxidization potential. The advantages of this method include good selectivity, low limits of detection, real-time monitoring and ease of handling.

The use of metals such as gold, silver, or platinum to electrochemically oxidize NO is hampered by slow electron transfer and the strong adsorption of NO to the electrode surface. Malinski and Taha first reported the electrocatalytic oxidation of NO at a carbon-based electrode which improves the selectivity and sensitivity for NO detection (35). They applied a nickel porphyrin film to a carbon fiber (1–20μm diameter) electrode, which lowers the oxidation potential for NO to approximately +0.65 V versus SCE (36). The microsensor, which can be operated in either the amperometric or voltammetric mode, had a low detection limit of 10 nM NO with a 0.1–10 ms response time. Since the pioneering work of Malinski and co-workers, the carbon fiber electrode is now among the most widely used method for NO detection.

1.2.3. The role of nitric oxide in cancer

Studies indicate that NO plays multifaceted roles (suppression vs. promotion) in cancer. Tumor cells are found to produce large amounts of NO via induced iNOS expression (37). In addition to iNOS, several tumor cell types also express eNOS or nNOS, while endothelial cells within the tumor express eNOS (37). Some reports suggest that NO produced endogenously by tumor cells may reduce their metastatic potential,
since the induction of NO synthase by cytokines in murine melanoma cells resulted in a dramatic decrease in lung metastasis in vivo (38). It has also been found that the decline in NO production in liver endothelial cells leads to progressive tumor growth and high load of liver metastasis (39), while NO produced in the brain vasculature limits the spread of colon cancer to the brain (40). In another study, lipopolysaccharide has been shown to inhibit melanoma metastasis in the liver through induction of NO and the expression of adhesion molecules on the hepatic endothelium (41). Furthermore, NO may suppress metastasis by interfering with the hetero-adhesion between cancer cells and human umbilical vein endothelium via down-regulation of cell adhesion molecules (42).

Several mechanisms accounting for tumor-suppressing effects of NO can be summarized as follows: a) exerting cytotoxicity towards tumor cells through inhibition of mitochondrial respiration and DNA replication in tumor cells (37,42); b) direct vasorelaxation which enlarges capillary diameter to prevent CTCs from trapping there (37,41); c) inhibition of VCAM and ICAM expression (42).

However, the tumor-suppressing role of NO is weighted by studies showing a tumor-promoting activity of NO. NO produced by tumor cells has been associated with enhanced murine mammary tumor cell invasiveness by altering the balance between matrix metalloproteinase-2 (a protein involved in the breakdown of extracellular matrix) and its inhibitors (44). Moreover, human carcinomas have elevated iNOS level and genetic deletion of iNOS has been shown to reduce tumor formation in several mouse models of cancer. For instance, genetic disruption of iNOS resulted in an 80% decreased in urethane-induced lung tumor formation in mice, suggesting iNOS inhibitors as
potential cancer chemopreventive agents (45). Similarly, iNOS-mediated NO formation was found to be positively correlated with gastric carcinogenesis and lymph node metastasis (46). Another study also indicates the involvement of NO and iNOS in increased invasion of human colorectal adenocarcinoma cell lines (47). NO may promote tumor formation and progression by stimulating tumor angiogenesis, by increasing tumor cell migration and invasion through guanylyl cyclase and MAPK signaling, or by inducing DNA mutations (37). Growing evidence indicates that DNA mutations associated with carcinogenesis may be caused by ONOO\(^-\) instead of NO (37).

In summary, the effects of NO may be mediated in part by one of its metabolites, peroxynitrite, which is a potent oxidizing agent and may regulate many of the carcinogenic effects as DNA damage, protein or lipid modification (48). Depending on the environmental milieu, NO and its byproduct ONOO\(^-\) can exert various effects on intracellular redox states and may function as an important regulator in cancer development.

1.2.4. Nitric oxide and oxidative stress

Oxidative stress takes place when reactive oxygen species (ROS) production exceeds the handling capability of intracellular and extracellular antioxidants (49). It is closely associated with cardiovascular diseases and endothelial dysfunction. ROS production is strictly regulated by vascular smooth muscle cells and the endothelium under normal physiological conditions (60). However, cellular superoxide levels can be chronically up-regulated by stimuli which leads to aberrant cell signaling and endothelial
dysfunction (50). High levels of superoxide react with NO to produce cytotoxic ONOO⁻ which can oxidize tetrahydrobiopterin (51) and reduces cellular transport of L-arginine (52), thus reducing NO bioavailability.

NO could prevent the focal adhesion of leukocytes through the inhibition of ICAM-1 and VCAM-1 expression on the endothelial cell surface (53). Initial studies suggested that NO may regulate cell adhesiveness by affecting cellular oxidative stress (59). Indeed, cell adhesion molecules are found to be closely associated with the cellular redox state (54). Intracellular ROS are necessary for integrin signaling during fibroblast adhesion (55). ROS can activate redox-sensitive transcription factors that regulate the expression of endothelial cell adhesion molecules (e.g., P-selectin) within the microvasculature (56). NO and oxidative stress are involved in the regulation of VCAM-1 gene expression, possibly through the reduction-oxidation sensitive NF-κB (57). Oxidative stress enhanced monocyte adhesion to HUVEC through induction of the gene expression of ICAM-1, E-selectin and monocyte chemoattractant protein-1 (MCP-1) (58). Another study indicated that oxidative stress may regulate VCAM-1 through specific reduction-oxidation sensitive transcriptional factors such as NF-κB (57). Moreover, antioxidants inhibit lipoprotein induced ICAM-1 and VCAM-1 expression on HUVECs (59). Similarly, antioxidants was found to inhibit monocyte adhesion by suppressing NF-κB activation and VCAM-1 expression in endothelial cells (61,62). In addition, hyperglycemia was found to stimulate oxidative stress and increase the expression of adhesion molecules (61).
1.2.5. Nitric oxide and cell adhesion

NO has been shown to mediate cell–cell interactions in the vasculature and growing evidence suggests that NO may be an anti-adhesive molecule. For example, S-nitrosocaptopril, a NO donor, was shown to decrease the adhesion of human colorectal cancer cells to HUVECs via down-regulation of VCAM-1 expression (42). In addition, NOS inhibitors have been found to increase leukocyte–endothelial interactions through the expression of platelet selectin (P-selectin), ICAM-1 and VCAM-1 in normal vessels (62). Furthermore, NO inhibits human platelet adhesion to vascular endothelium (63). NO has also been shown to decrease neutrophil adhesion to postcapillary venular endothelium (64).

Pro-inflammatory factors such as TNF-α, interleukin-1, and lipopolysaccharide have been shown to increase tumor cell adhesion to endothelium by increasing the expression of adhesion molecules such as ICAM-1 and VCAM-1 on both cell types (65). VCAM-1 expression induced by TNF-α requires the activation of NF-κB through the phosphorylation and degradation of its cytoplasmic inhibitor, IkappaBalpha (66). NO has been shown to decrease VCAM-1 expression by inhibition of NF-κB activation and increasing the expression and nuclear translocation of IkappaBalpha (66). Similar results also indicated that NO donors increased the expression of ICAM-1 on a squamous cell carcinoma cell line via the NF-κB signaling pathway, a process which was abolished in the presence of carboxy-PTIO, a NO scavenger (67).
1.2.6. Nitric oxide and shear stress

Shear stress is a dragging mechanical force acting at the interface between the endothelium and blood flow (68). Shear stresses exerted by flowing blood and interstitial fluid regulate the behavior of circulating tumor cells (CTCs) and endothelial cells (30,63). Increasing shear stresses may damage CTCs (69) and increases the amount of collisions of CTCs with vessel walls, but it also reduces their residence time of adhesion (31). It is also probable that interstitial fluid flow may cause changes in local concentration gradients of signaling proteins which in turn may regulate cancer cell responses to ECs (70). Overexpression of growth factors such as VEGF-C and VEGF-D in tumors might result in vessel dilation and increased volumetric flow rate (71). The lymph fluid velocity in tumors is about an order of magnitude higher than the interstitial fluid velocity (72). Increased lymph flow rate might lead to a 200-fold increase in the accumulation of cancer cells in lymph nodes and a 4-fold increase in metastasis (73). Furthermore, an increase in interstitial flow can increase lymphatic permeability which may increase the chance of CTCs to enter the lymphatic vessels (31). Therefore, lymph flow plays a crucial role in the dissemination of cancer cells (31).

Arterial and arteriolar endothelial cells control blood flow through release of autacoids such as NO and endothelium-derived hyperpolarizing factor (EDHF) (36). NO plays a dominant role in vasorelaxation especially in large arteries, whereas EDHF may predominate in smaller vessels (74). NO released by the vascular endothelium is a powerful vasodilator that plays a role in the physiological regulation of blood flow and blood pressure (75). Inhibition of NOS blocked the increase in blood flow rate (76).
Thus, dysfunctional endothelium failing to generate adequate amounts of bioavailable NO causes a profound impact on NO-mediated vasorelaxation (49). Inhibition of eNOS was also found to decrease lymphatic fluid velocity of the deeper collecting lymphatics, while the superficial lymphatics did not change with eNOS inhibition (31). Impaired vasodilatation, commonly referred to as endothelial dysfunction, is frequently associated with inflammation and elevated cytokine levels. Cytokine such as TNF and IFN-γ rapidly induce EC to synthesize tetrahydrobiopterin, a cofactor for eNOS, to increase NO production (77), while these cytokines diminish NO release by destabilizing eNOS transcripts at later times (78). Inflammatory cytokines may also reduce NO bioavailability by enhancing superoxide production, which inactivates NO (79). TNF can further inhibit endothelial NO production by reducing protein kinase B (Akt)-mediated phosphorylation of eNOS (38,39), thereby reducing NO-induced vasodilation.

1.3. Peroxynitrite

1.3.1. The role of peroxynitrite in biological system

Peroxynitrite is a reactive oxidant generated from nitric oxide and superoxide anion that regulates a variety of biological processes, including inhibition of leukocyte adhesion, blockage of platelet aggregation, and induction of vasodilation in mammalian cells, apoptosis and necrosis (81). Despite a relatively short half-life (~10 ms), peroxynitrite can oxidize target molecules either directly or indirectly (82). ONOO⁻ directly and rapidly oxidizes sulfhydryl groups and initiates lipid peroxidation (46). Alternatively, ONOO⁻ can cause the formation of nitrotyrosine which can serve as a
ONOO⁻ "footprint" (83). Some of these actions might lead to irreversible tissue damage (75).

Peroxynitrite also interacts with DNA through nitrosative and oxidative modifications of nucleobases and by producing single-strand DNA breaks, both of which have been associated with greater risk of cancer (84). In addition, ONOO⁻ may inactivate DNA repair enzymes such as 8-oxoguanine DNA glycosylase, or by inhibiting the transcription factor p53 through tyrosine nitration (85). The former is a key enzyme responsible for the base excision repair of 8-oxoguanine, a product of the oxidation of guanine by ONOO⁻, while the latter is a tumor suppressor protein.

Recent evidence indicates that increased ONOO⁻ production within endothelium is probably the major reason for the cytotoxicity of NO (37). NAD(P)H-dependent oxidases, xanthine oxidase, cyclooxygenase, mitochondrial oxidases, eNOS and nNOS have been suggested as the source of O₂⁻ (86). Inactivation of endothelium-derived NO by O₂⁻ to form peroxynitrite is responsible for endothelial dysfunction. ONOO⁻ may cause tyrosine nitration and oxidation of various proteins including eNOS, resulting in decreased NO production (49).

1.3.2. Detection of peroxynitrite

Peroxynitrite is a highly reactive species with a short half-life (~1s). It is biologically generated by the reaction of NO and O₂•⁻ through the following reaction:

\[
\text{NO} + \text{O}_2 \cdot^- \rightarrow \text{ONOO}^{-}
\]
Monitoring ONOO\textsuperscript{−} in biological systems could help understand its role in different diseases (87). Similar to the detection of NO, an electrochemical method is most likely the best option for ONOO\textsuperscript{−} detection since ONOO\textsuperscript{−} oxidizes at a modest potential (88). In addition, it is possible to detect many other electroactive species as well since individual species exhibit different voltammetric profiles. By now there are several sensors available for detecting ONOO\textsuperscript{−} released from several types of cells, these sensors are mostly carbon-based electrodes (88,89).

1.3.3. The role of peroxynitrite in cancer

Peroxynitrite is known to cause DNA damage mainly through guanine modifications, induction of DNA single-strand breaks and the inhibition of DNA repair enzymes. Peroxynitrite triggers oxidation and nitration of guanine to form 8-oxoguanine and 8-nitroguanine respectively, leading to potential mutations in DNA (G to A, G to T, and G to C mutations) (37). In addition, peroxynitrite can produce DNA single-strand breaks by cleavage of abasic sites or oxidation of the deoxyribose moiety (62). The guanine oxidation and single-strand breaks induced by peroxynitrite have been identified as a critical step in carcinogenesis. Peroxynitrite may also cause inactivation of DNA repair enzymes and p53, an important factor to remove cells with significant DNA damage (37).

The formation of 8-nitroguanine \textit{in vivo} under chronic inflammatory conditions suggest that nitrative DNA damage consecutive to peroxynitrite overproduction might promote cancer development (37). It has been suggested that ONOO\textsuperscript{−} may contribute to
tumor progression by irreversibly weakening Arylamine N-acetyltransferase 1, a key cellular defense enzyme expressed in MCF7 breast cancer cells (90). Another study indicated that treatment with the peroxynitrite decomposition catalyst in mice reduced the spontaneous development of intestinal polyps, which is closely associated with the development of colon carcinoma (37).

1.3.4. Peroxynitrite and cell adhesion

Accumulating evidence suggests that ONOO\(^{-}\) formation plays an important role in cell adhesion. Nanomolar concentrations of ONOO\(^{-}\) were found to inhibit leukocyte-endothelial cell interactions through the inhibition of P-selectin expression on the endothelial cell surface (91). It is also found that ONOO\(^{-}\) increased endothelial VCAM-1, P-selectin, and E-selectin expression, which may enhance neutrophil-endothelial interaction (92). Another study showed that addition of ONOO\(^{-}\) at micromolar concentrations resulted in down-regulation of L-selectin and up-regulation of CD11/CD18 expression on human neutrophils via the Ras/Raf-1/MEK signaling cascade, leading to increased neutrophil adhesion to human coronary artery endothelial cells (93). In addition, ONOO\(^{-}\) decomposition catalysts were capable of exerting a protective effect in a model of splanchnic artery occlusion shock through inhibition of the expression of adhesion molecules (94). Given the similarity between cell-cell adhesions, it is tempting to speculate that ONOO\(^{-}\) is involved in mediating tumor cell-endothelial cell interaction as well.
1.3.5. Peroxynitrite and shear stress

Peroxynitrite may regulate shear stresses through inhibition of vasodilatation. A wide range of concentrations of ONOO$^-$ has been found to induce vascular dysfunction and inhibit the vasodilatation of the coronary vasculature in the isolated perfused rat heart (75). Others found that ONOO$^-$ caused vasoconstriction in rat lungs by a process involving protein synthesis and engagement of the receptors for the extremely potent vasoconstrictor ET-1 (95). In another study, the repeated administration of peroxynitrite was found to increase arterial pressure and vascular resistances in anesthetized rats, indicating that peroxynitrite production may contribute to hypertension in vivo (96). Similarly, the systemic administration of peroxynitrite has been found to attenuate the vasodilatory effect of prostacyclin, a vasodilator, through inhibition of vascular smooth muscle ATP-sensitive potassium channel activation (97). Additionally, the administration of ONOO$^-$ increases hindquarter, renal, and mesenteric vascular resistance in a time-dependent manner, a process which may result from attenuation of endothelium-dependent vasorelaxation and concomitant loss of β-adrenoceptor-mediated vasodilatation (98). ONOO$^-$ is a strong oxidant which can initiate lipid peroxidation, sulphydryl oxidation, and nitration of aromatic amino acids, leading to irreversible tissue damage and impaired function in the coronary circulation (75).

However, a number of other studies showed that ONOO$^-$ caused vasodilatation of the vasculature. For instance, ONOO$^-$ was found to dose-dependently decrease arterial pressure and vascular resistance in rats (98). Another study indicates ONOO$^-$ as a less potent vasorelaxant species compared with NO and it induced vasorelaxation in canine
coronary arteries (99). Similarly, the vasodilatory effect of ONOO$^-$ was found to be around 50-fold less than that of NO (100). ONOO$^-$ may induce vasodilatation through activation of ATP-sensitive potassium channels in vascular smooth muscle (101); or via generation of NO by secondary reactions. For example, ONOO$^-$ can be oxidized to produce ONOO$^-$, which is in equilibrium with NO and O$_2$ (99). ONOO$^-$ can also be converted to NO donors such as S-nitrosothiols in the presence of low concentrations of human plasma, serum albumin, or glutathione, which may subsequently release NO (75). It is also been suggested that ONOO$^-$ causes vascular relaxation through nitrosylation of tissue thiol groups which subsequently release NO (100). Thus, the formation of NO may be responsible for the vasodilatory effects of ONOO$^-$ in the vasculature. However, the vasodilatory effects of ONOO$^-$ are subject to the development of rapid tachyphylaxis, which attenuates vascular relaxation after development of tachyphylaxis to ONOO$^-$ in vivo (97). Moreover, at high concentrations of ONOO$^-$ its damaging effects may overcome the protective effects of the NO generated (75). Thus, whether ONOO$^-$ would cause vasodilatation or vasoconstriction may depend on several factors such as its concentration in the microenvironment, the rate of its conversion to NO donors and the extent of ONOO$^-$ induced tissue injury (75).

1.4. Adhesion molecules in cancer metastasis

Increasing evidence suggests that the expression of specific cell surface molecules are responsible for tumor cell adhesion to the endothelial cells (102). In response to specific mediators, vascular endothelial cells express a handful of cell surface molecules
capable of supporting cancer cell adhesion. If the number of molecules is sufficient, the physical conditions such as shear forces are permissive, and the cancer cells are responsive, adhesion may occur (10). Until now, researchers have identified more than a hundred cell adhesion molecules which are divided into five categories; cadherins, integrins, immunoglobulin gene superfamily (IgSF), selectins, and CD44 (103).

1.4.1. Cadherins

Cadherins mediate cell adhesion in a calcium-dependent manner. The major members of the cadherin family include E-cadherin, N-cadherin and P-cadherin. Among them, E-cadherin is mostly involved in cancer development since more than 90% of human carcinomas arise from epithelial tissues. Downregulation of E-cadherin has been found in many types of human cancers. For example, studies examined the expression of E-cadherin in primary and metastatic gastric carcinoma and suggested that down-regulation of E-cadherin is a common early event in gastric cancer (104). In another study, E-cadherin expression was analyzed in nonmalignant and malignant specimens of human prostate cancer. Around 50% of either primary or metastatic prostate tumor samples had reduced or absent E-cadherin expression when compared to nonmalignant tissues (105), suggesting a correlation between loss of E-cadherin expression and tumor progression and that E-cadherin expression can serve as a marker for metastatic prostate cancer. Loss of E-cadherin expression in tumors was also found to be associated with high grade and advanced stage in pancreatic cancer (106).
1.4.2. Integrins

Integrins are heterodimers composed of α and β subunits and their expression has been found in almost every cell/tissue studied, most predominantly leukocyte surface (103). Nowadays, at least nine α subunits and fifteen β subunits have been identified (103). Integrins are receptors for a variety of proteins including fibronectin, fibrinogen and the immunoglobulin superfamily such as ICAM-1 and VCAM-1, thus play a key role in cell adhesion, proliferation, and migration (107). Down-regulation of integrin expression has been found in several types of cancer, such as prostate cancer. It has also been found that the adhesion of melanoma cells to lymph node vitronectin via the αvβ3 receptor plays an important role in lymph node metastasis (108). Also, activated integrin αvβ3 expressed on metastatic human breast cancer cells supported breast cancer cell attachment under blood flow conditions and strongly promoted metastasis in the mouse model (109). These results indicate that the use of anti-integrin antibodies may be potential treatment regimens for specific cancers.

1.4.3. Immunoglobulin gene superfamily

Members of the immunoglobulin gene superfamily were found to be involved in tumor spread. The most important members of the family include ICAM-1, ICAM-2 (Intercellular Adhesion Molecule-2), VCAM-1, PECAM-1 (Platelet Endothelial Cell Adhesion Molecule-1), MAdCAM-1 (Mucosal Addressing Cell Adhesion Molecule-1), NCAM (Neural Cell Adhesion Molecule), and CEA (CarcinoEmbryonic Antigen).
Approximately 50% of the surface glycoproteins of leukocytes belong to the immunoglobulin superfamily (103).

Endothelial cells express an array of molecules such as ICAM-1, ICAM-2, VCAM-1, and PECAM-1 that belongs to the immunoglobulin superfamily and participate in cell adhesion (25). ICAM-1 is a 90 kD transmembrane glycoprotein and cells known to express which is expressed on the surface of many cell types including endothelial cell, epithelial cells, lymphocytes, monocytes, and fibroblasts. The most important ligands for ICAM-1 are the β2 integrins LFA 1 and Mac-1 that are expressed on leukocytes. VCAM-1 is another transmembrane glycoprotein of 100-110 kDa which is expressed on the surface of neurons, endothelial cells, smooth muscle cells, fibroblasts and macrophages. Endothelial ICAM-1 and VCAM-1 expression is at low levels under normal conditions but is dramatically upregulated by endotoxin and cytokines such as IL-1β, IL-4 and TNF-α (110). Single tumor cells adhere to the endothelium via cell adhesion receptors like integrins or immunoglobulins and their counter-receptors on endothelial cells (111). The cytokine-inducible VCAM-1 found on the lumenal surface of endothelial cells serves as a ligand for the α4β1 integrin (VLA-4) expressed on several tumour cell types, including rhabdomyosarcoma, neuroblastoma and melanoma. Growing evidence indicates that VCAM-1 mediates cancer cell adhesion through an interaction with the α4β1 integrin (112). For instance, mice pre-treated with cytokines have elevated VCAM-1 expression on endothelial cells and increased metastatic tumor load resulting from the injection of α4β1-expressing melanoma lines of either murine or human origin (102).
Inducible cell adhesion molecule 110 (INCAM-110), another glycoprotein found on the surface of cytokine-activated endothelial cells, was found to mediate the adhesion of metastatic melanoma cell lines, a process which was inhibited by the anti-INCAM-110 monoclonal antibody (102). Other endothelial leukocyte adhesion molecules may also participate in the metastatic process (10). These findings highlight the importance of cell adhesion molecules in the interaction between tumor cells and endothelial cells and draw attention to the possibility of targeting cell adhesion molecules in metastatic cancer.

1.4.4. Selectins

The selectins consist of three well-characterized members including E-selectin, P-selectin and L-selectin which bind to carbohydrate and are associated with extravasation of leukocytes or tumor cells in inflammation and cancer metastasis (113). E-selectin and P-selectin are expressed on endothelial cells and L-selectin is expressed on leucocytes. Previous studies indicate that the interactions of endothelial selectins with glycoconjugates on tumor cells plays an important role in tumor cell dissemination (114). For example, sialylated-Lewis x (sLex) or sialyl-Lewis a (sLea) expressed on human colon cancers appear to participate in this adhesive process through interaction with E-selectin (115). Additionally, more than 80% reduction in spontaneous lung metastasis formation from human colon cancer has been observed in E- and P-selectin-deficient mice, which highlights the importance of selectins in spontaneous metastasis formation (114). In sum, agents which target cellular interactions with members of the selectin family may be potential therapeutics in controlling cancer metastasis (113).
1.4.5. CD44

CD44, a 85-95 kDa cell membrane glycoprotein, has been the subject of extensive research because of its role in breast cancer as well as its utility as a stem cell marker (116). The role of CD44 in cancer biology is controversial. Most human cancers have dysregulated CD44 expression (117). Additionally, the expression of different forms of CD44 (standard form vs. variant form) on tumor cells has been correlated with their metastatic potential. The variant form of CD44 was expressed only on the metastatic cells. For example, CD44v6, a member of the CD44 family, has been identified as metastatic determinants of pancreatic tumor cells in rats. In fact, antibodies targeting CD44v6 isoforms has been used in clinical trials because of their frequent expression in squamous cell carcinoma (118).

1.5. Aim of work

Summarizing the previous studies, no study has evaluated the association between the cytoprotective NO and cytotoxic ONOO\(^-\) balance and lymphatic metastasis. Therefore, we conducted this study to identify the role of \([\text{NO}]/[\text{ONOO}^-]\) balance in cancer cell-lymphatic endothelial cell interaction, with particular attention to CACO-2 colorectal and HT1080 fibrosarcoma cancer cells.

We hypothesized that nitrooxidative/oxidative stress would cause endothelial dysfunction and promote cancer cell adhesion. To test this hypothesis, we introduced a ratio of NO and ONOO\(^-\) concentration \([\text{NO}]/[\text{ONOO}^-]\) as a marker for dysfunctional endothelium. Using the electrochemical porphyrinic sensors, NO and ONOO\(^-\) release
from lymphatic endothelial cells was measured in vitro during cancer cell adhesion under static and flow conditions. A lab designed parallel flow chamber coupled with electrochemical nanosensors was applied to measure NO and ONOO$^-$ concentrations under flow condition.

We further investigated several NO and ONOO$^-$ modulators on their potential to regulate cancer cell-lymphatic endothelium interaction. To study the mechanism of how NO and ONOO$^-$ regulate cancer cell adhesion, ICAM-1 and VCAM-1 expression on non-stimulated and TNF-α stimulated HLEC was examined by ELISA.

In order to determine the metastatic potential of different cancer cell lines, we compared the adhesion patterns of three cancer cell lines (Caco-2, HT1080, MCF-7) to HUVECs and HLECs under static and flow conditions.
2. Materials and Methods

2.1. Cell cultures

Human lymphatic endothelial cells (HLECs) and Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from Sciencell and grown in complete endothelial cell growth medium according to the supplier’s suggestions. Cells from passage 1 or 8 were used in the experiments. CACO-2, HT1080 and MCF-7 cells were obtained from ATCC and maintained in culture medium (Eagle’s Minimum Essential Medium containing 10% fetal bovine serum). The cells were maintained in 95% air/5% CO₂ at 37°C.

2.2. Preparation of NO electrode

NO release from endothelial cells was monitored by a porphyrinic electrode, which oxidizes NO to produce an electrical signal proportional to NO concentration. As shown in Figure 2.1, a few carbon fibers and a copper wire were inserted into a glass capillary (35). The tip of the carbon fibers was sharpened by flame to obtain a diameter of 300-500 nm. The electrode was then coated with a polymeric film through electrochemical polymerization of nickel(II) tetrakis (3-methoxy-4-hydroxy-phenyl) porphyrin (TMHPPNi) (Mid-century Chemicals, USA) by cyclic voltammetry. The outer layer of the carbon fiber is covered with negatively charged Nafion (Aldrich, USA), which repels anions and is highly permeable to NO.

NO release was measured in a three electrode system which consists of a NO working electrode, a Ag/AgCl reference electrode and a platinum counter electrode. The
electrode works in an amperometric mode, in which a fixed potential is applied between the NO working electrode and the reference electrode. All measurements were performed in a Faraday's cage to reduce noise.

Figure 2.1 Structure of the nitric oxide nanosensor (Adapted and modified from reference (35)).

Figure 2.1 Structure of the nitric oxide nanosensor (Adapted and modified from reference (35)).

2.3. Preparation of ONOO⁻ electrode

The preparation of the ONOO⁻ nanosensor is similar to that of the NO sensor (Figure 2.2). Carbon fibers are pulled through a glass capillary and electrochemically coated with a film polymerized from Mn(II)mesotris(N-methyl-4-pyridinium)-p-
phenylene-5.-O-2.,3.-sopropyldiene uridineporphyrin (MnPUP) (Mid-century Chemicals, USA) by cyclic voltammetry. The sensor works in an amperometric mode to produce a current signal which is linearly proportional to ONOO\(^{-}\) concentrations. The sensors were calibrated with ONOO\(^{-}\) standard solutions after the measurements.

Figure 2.2 Structure of the peroxynitrite nanosensor (Adapted and modified from reference(119)).
2.4. Measurement of NO and ONOO− concentrations using nanosensors

Measurements of NO and ONOO− concentrations were carried out with electrochemical nanosensors with a total diameter of 200–500nm and length of 4–5 mm, as previously described (72,73). Each nanosensor was coated with a conductive polymeric film. The NO sensor was coated with polymeric nickel (II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin and the ONOO− sensor was coated with a polymeric film of Mn (III)–paracyclocophanyl-porphyrin. Each sensor was calibrated with NO or ONOO− standard solutions before measurements.

Cancer cells were dissolved in EMEM basal medium to obtain different concentrations. Under static condition, NO and ONOO− release was stimulated by cancer cells injected with a nanoinjector that was positioned by a computer-controlled micromanipulator. Under flow condition, NO and ONOO− release was measured with a lab designed parallel flow chamber as previously described (119). The flow chamber was coupled with electrochemical nanosensors and connected to a syringe pump (model KDS210, kdScientific Inc., Holliston, MA) which draws assay medium through. The sensor was positioned above the surface (5 ± 2mm) of a randomly chosen single endothelial cell. Four to six measurements were performed per group.

2.5. Static adhesion assay

HLECs and HUVECs were cultured in a flat-bottom 96 well plate. The plates were incubated at 37°C, 95% relative humidity, 5% CO2. The HLECs reached confluence in 2 to 3 days as determined by light microscopy. Before the static adhesion assay, HLEC
monolayers were pretreated for 30 minutes, 1 hr or 2 hrs at 37°C with various chemicals (mM): 3-morpholinosydnonimine HCl (SIN-1, Sigma-Aldrich), a releaser of both NO and O$_2$; NG-Nitro-L-Arginine-Methyl ester (L-NAME, Sigma-Aldrich), an eNOS inhibitor; L-arginine (Sigma-Aldrich), Superoxide dismutase–polyethylene glycol (PEG-SOD, Sigma-Aldrich), N-(acetyloxy)-3-nitrosothiovaline (SNAP, Cayman Chemical), VAS2870 (Sigma-Aldrich) or Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP, Cayman Chemical), a cell-permeable superoxide dismutase (SOD) mimetics. In some experiments, HLEC monolayer was pre-incubated with 1 ng/ml recombinant TNF-α (R&D Systems) for 24 hrs prior to drug treatment.

To quantify cell adhesion, the tumor cells were labeled with 1 μM CellTracker™Green CMFDA (Molecular Probes) for 45 minutes at 37°C with occasional mixing, washed 2 times and added to the confluent endothelial monolayers at 2 x 10$^5$ cells/well. Tumor cells were allowed to adhere for 30 min at 37°C. Thereafter, the wells were washed 3 times with DPBS (Dulbecco's Phosphate-Buffered Saline) to remove non-adherent cells. The remaining mean fluorescence intensities (MFI) was measured on a plate reader using 485 nm excitation and 530 nm emission filters.

2.6. Flow adhesion assay

Cancer cell interactions with HLECs were quantified under physiological flow conditions with a flow chamber. The flow chamber consisted of 35-mm tissue culture dish bearing a HLEC monolayer and a flow deck which were kept together by vacuum. The flow chamber was connected to a syringe pump (model KDS210, kdScientific Inc.,
Holliston, MA) to draw assay medium through. A flow section of 0.0125-cm thickness and 0.5-cm width was formed between the flow chamber deck and culture dish, as determined by a silicone gasket. The endothelial cells were exposed to laminar shear stress (τ) of 0.1 to 0.8 dyne/cm² by changing the flow rate using following formula.

\[ \tau = \frac{6\mu Q}{wh^2} \]

Where μ is the media viscosity (0.008 g/cm·s), w is the channel width (0.5 cm), h is the channel height (0.0125 cm), and Q is the volumetric flow rate.

The HLEC monolayer was pre-incubated with recombinant TNF-α (50ng/ml) for 24 hrs prior to drug treatment. Adhesion assays were performed by perfusing cancer cells at a fixed concentration of 1x10⁶/ml in EMEM, at the wall shear stresses of 0.1–1.0 dyne/cm², which mimics the microcirculation environment (<3dynes/cm²) (121). For a 1-minute stabilization period, EMEM was drawn over the endothelial monolayer at 0.1dyne/cm². Thereafter the valve was switched and cancer cells suspended in the EMEM medium were perfused over the HLEC monolayer. The total numbers of adherent cells during the 10-min perfusion period were quantified by manually reviewing the videotaped experiments. Representative pictures of three different fields were used to determine the amount of adherent cells under flow condition. All experiments were repeated three times. Results are shown as mean ±S D.

2.7. ELISA

Expression of adhesion molecules on the surface of HLECs was determined by ELISA. HLECs were plated in 96-well plates and maintained until confluent. Following
incubation with various chemicals for the required periods at 37°C, the wells were fixed with 4% formaldehyde and then treated for 1h at room temperature with 2% BSA/Phosphate-Buffered Saline with Tween 20 (PBST) to block non-specific binding. Appropriate amounts of mAbs against ICAM-1 and VCAM-1 (Santa Cruz Biotechnology, Inc., CA) were added and the HLECs were incubated at 4°C overnight, washed with assay medium, treated at room temperature for 1 h with the second mAb (HRP-conjugated goat anti-mouse IgG; Santa Cruz Biotechnology, Inc., CA). After color was developed by adding 3,3’5,5’-tetramethylbenzidine for 10 minutes, the reaction was terminated with 2M citric acid. Plates were then read in a BioTek plate reader at 450nm.

2.8. Statistics

All values were expressed as means ± SD representing at least three separate experiments. Two-tailed Student’s t-test were used to compare the mean difference between two groups. The α level for all tests was 0.05. A P value <0.05 was considered significant. GraphPad Prism software version 6 was used in the statistical analyses.
3. Results

3.1. Static adhesion

3.1.1. NO and ONOO\(^{-}\) release from endothelium during tumor cell adhesion

We performed limited studies of the adhesion of cancer cells to HUVECs only as a background study to compare it with the adhesion to HLEC. The original full studies of adhesion of cancer cells to HUVECs were performed in this lab by Feng Liu (119).

Using chemical nanosensors, we measured NO and ONOO\(^{-}\) release from HLECs and HUVECs following the administration of CACO-2, HT1080 or MCF-7 cells over a range of concentrations. Figure 3.1 shows a typical recording of NO and ONOO\(^{-}\) concentration versus time obtained from a single HLEC after injection of HT1080 cancer cells. The peak concentration stimulated by HT1080 represents the maximum NO or ONOO\(^{-}\) concentration that can be reached from a single HLEC. The concentration-dependent peak NO release for all three cancer cell lines is shown in Figure 3.2A. There is a linear increase in NO release with CACO-2 stimulation in the range of concentration between 0.625 and 5x10\(^5\)cells/ml. The peak NO release from HLECs after injection of the lowest concentration (0.625x10\(^5\)cells/ml) of CACO-2 cells was 115±7nM. When CACO-2 concentration was increased to 5x10\(^5\)cells/ml, NO production was augmented almost three fold to 545 ± 21nM, which was the highest among the three cancer cell lines tested. At concentrations of CACO-2 1x10\(^6\)cells/ml, a decline in NO release was observed. Similar trend was observed for HT1080 and MCF-7.

Similarly, an increased ONOO\(^{-}\) production was observed when cancer cell concentration was increased (Figure 3.2B). At 1x10\(^6\)cells/ml, ONOO\(^{-}\) release from
HLECs was the highest with CACO-2 cell stimulation (319±23nM) and the lowest with MCF-7 cell stimulation (230±5nM).

We also measured NO and ONOO⁻ release from HUVECs, another endothelial cell type, following cancer cell stimulation. The concentration-dependent peak NO release for all three cancer cell lines is shown in Figure 3.3A. There is an increase of NO production with CACO-2 cell stimulation in the range between $0.625 \times 10^5$ and $1 \times 10^6$ cells/ml. The peak NO release from HUVECs after injection of the lowest concentration of CACO-2 cells ($0.625 \times 10^5$ cells/ml) was 67±7nM. NO production was augmented almost three fold to 243±27nM when CACO-2 concentration was increased to $1 \times 10^6$ cells/ml. A similar trend was observed with HT1080 cell stimulation. The peak NO release from HUVECs after injection of the lowest concentration ($0.625 \times 10^5$ cells/ml) and the highest concentration ($1 \times 10^6$ cells/ml) of HT1080 cells was 63±5nM and 245±30nM respectively. At a concentration of $1 \times 10^6$ cells/ml, MCF-7 produced the lowest amount of NO (100±9nM) among the three cancer cell lines.

Similarly, an increased ONOO⁻ production was observed for all three cancer cell lines with increasing amount of cancer cell stimulation (Figure 3.3B). The peak ONOO⁻ release from HUVECs after injection of the lowest concentration ($0.625 \times 10^5$ cells/ml) of CACO-2, HT1080 and MCF-7 cells was 74±4nM, 111±4nM and 67±7nM respectively. At $1 \times 10^6$ cells/ml, ONOO⁻ concentration was the highest with CACO-2 stimulation (235±30nM) and the lowest with MCF-7 stimulation (131±26nM) (Figure 3.3B).
Figure 3.1 Typical amperograms of NO and ONOO$^-$ release recorded *in vitro* on the surface of a single human lymphatic endothelial cell. The release of NO and ONOO$^-$ was stimulated by HT-1080 human fibrosarcoma cells (2.5x10$^5$ cells/ml) injected with a nanoinjector that was positioned by a computer-controlled micromanipulator and measured *in situ* by electrochemical nanosensors.
Figure 3.2 Maximal NO (A) and ONOO\(^-\) (B) concentration release from HLECs stimulated with different concentrations of cancer cells.

The release of NO and ONOO\(^-\) was stimulated by 5\(\mu l\) of different concentrations of cancer cells (0, 0.625, 1.25, 2.5, 5 and 10\(x10^5\)cells/ml) on a 96 well plate and measured \textit{in situ} by electrochemical nanosensors. N=3-6. Two-tailed Student’s t-test:*P<0.05 compared with 0.625\(x10^5\)cells/ml.
Figure 3.3 Maximal NO (A) and ONOO$^-$ (B) concentration release from HUVECs after stimulation with different concentrations of cancer cells. The release of NO and ONOO$^-$ was stimulated by 5µl of different concentrations of cancer cells on a 96 well plate and measured in situ by electrochemical nanosensors. N=3-4. Two-tailed Student’s t-test: *P<0.05 compared with 0.625x10$^5$ cells/ml.
Figure 3.4 The [NO]/[ONOO⁻] ratio calculated from the maximal NO and ONOO⁻ concentration in HLEC (A) and HUVEC (B). Values are mean ± S.D. (N=4-6). Two-tailed Student’s t-test:*P<0.05 compared with 5x10⁵ cells/ml.
3.1.2. Cancer cells induce an imbalance of \([\text{NO}] / [\text{ONOO}^-]\) in endothelium

We used the ratio of NO to ONOO\(^-\) concentration to quantify an imbalance between NO and cytotoxic ONOO\(^-\) in the endothelium. A low \([\text{NO}] / [\text{ONOO}^-]\) ratio indicates a low concentration of the cytoprotective NO and/or a high level of cytotoxic ONOO\(^-\) (48). As shown in Figure 3.4A, the exposure of HLECs to a high concentration of cancer cells (higher than \(5 \times 10^5\) cells/ml) induced an unfavorable shift of the \([\text{NO}] / [\text{ONOO}^-]\) ratio to low levels for all three cancer cell lines. Indeed, CACO-2 cells induced a greater decrease in \([\text{NO}] / [\text{ONOO}^-]\) ratio in HLECs than an equal amount of HT1080 and MCF-7 cells. In HLECs, there is a significant difference in the \([\text{NO}] / [\text{ONOO}^-]\) ratio among CACO-2 (1.21±0.12), MCF-7 (1.39±0.20) and HT1080 (0.79±0.14) at a concentration of \(1 \times 10^6\) cells/ml. The ratio induced by HT1080 cells was below 1.0 indicating that the cellular environment is dominated by high oxidative/nitroxidative stress. Similar trends were observed in HUVECs. For all three cancer cell lines, there was a slight increase in the \([\text{NO}] / [\text{ONOO}^-]\) ratio in the range of concentration between 0.625 and \(2.5 \times 10^5\) cells/ml. However, the \([\text{NO}] / [\text{ONOO}^-]\) ratio declined significantly when cancer cell concentration was increased above \(2.5 \times 10^5\) cells/ml. At \(1 \times 10^6\) cells/ml, the ratio of \([\text{NO}] / [\text{ONOO}^-]\) for CACO-2, HT1080 and MCF-7 was 1.04±0.18, 1.17±0.18 and 1.22±0.33 respectively (Figure 3.4B). These results suggest that high oxidative/nitroxidative stress was induced to the cellular environment when cancer cell concentration increases.
Figure 3.5 Representative amperograms showing the changes of NO (upper curves) and ONOO⁻ (lower curves) concentration with time, recorded by nanosensors from a single human lymphatic endothelial cell after stimulation with $1 \times 10^6$ cells/ml of CACO-2 (a), HT-1080 (b), and MCF-7 (c).
3.1.3. Time course of the [NO]/[ONOO\'\] ratio in HLEC

Apart from examining the initial effect (approximately the first 1 min) of cancer cell stimulation on HLEC NO and ONOO\' release, a long-term effect (10 minutes) was also investigated. A distinctive difference between the slope and peak height of amperograms was observed for both NO and ONOO\' production (Figure 3.5). The slope of amperograms was used to calculate the rate of endothelial NO and ONOO\' release. A large increase in NO production was observed immediately after cancer cell stimulation.
The kinetics of NO release was relatively fast with CACO-2 stimulation, with a rate of 20±3nM/s, and significantly slower with MCF-7 (3.5±0.005nM/s) and HT-1080 (3±0.1nM/s) stimulation. The decay of NO concentration was significantly faster for CACO-2, with a rate of 244±8nM/s, and much slower for MCF-7 (13±0.1nM/s) and HT-1080 (29±1.6nM/s). The rates for ONOO⁻ release appear to have an opposite pattern to NO – lowest rate for CACO-2 (190±24nM/s) and higher for MCF-7 (464±65nM/s) and HT-1080 (305±51nM/s).

Figure 3.6 shows the changes in the [NO]/[ONOO⁻] ratio with time after the addition of 1x10⁶ cells/ml cancer cells. The [NO]/[ONOO⁻] ratio reached a maximum value within the first minute followed by a rapid decay for all three cancer cell lines. The [NO]/[ONOO⁻] ratio reached a plateau (lower than 1) after 480s indicating that the cellular environment is dominated by high oxidative/nitrooxidative stress.

3.1.4. The adhesion of cancer cells on HLEC and HUVEC is time-dependent and cell concentration-dependent

Figure 3.7A showed that the exposure of HLECs to higher amount of CACO-2 cells resulted in increased adhesion. There was no difference in cell adhesion in the first 20 min. However, a significantly higher amount of adhesion was observed with higher cell concentration after 30 min. While higher concentration of HT1080 cells showed a dramatically higher amount of adhesion through the entire time course under study. Similarly, the adhesion of CACO-2 and HT1080 cells on HUVEC also increased with
cell concentration (Figure 3.8). Therefore, the concentration of $1 \times 10^6$ cells/ml was used for all the experiments to ensure maximum adhesion under static condition.

3.1.5. Cancer cells show different affinity towards HUVEC and HLEC

To study the rate of adhesion, cancer cells were labeled with a fluorescent dye CMFDA and seeded on top of a confluent monolayer of endothelial cells. At various time points the non-attached cells were removed and the remaining firmly attached cells were analyzed. The results showed that cancer cell adhesion to the HLEC monolayer was relatively fast and time dependent. Among the three cancer cell lines, HT1080 has the highest rate of adhesion while the rate of MCF-7 adhesion is the lowest. Only a few cancer cells were attached to the endothelial monolayer within the first minute. However more attachment was seen after 10min. This is consistent with our time course study of the [NO]/[ONOO•] ratio revealing that sustained low levels of [NO]/[ONOO•] under chronic conditions lead to increased nitrooxidative/oxidative stress and cancer cell adhesion, which may contribute to malignancy.

Our data also showed that each of the three cancer cell lines has its own distinct pattern of relative adhesion to HLEC and HUVEC. Of the three cell lines, HT1080 shows a marked preferential adherence to HLEC compared to HUVEC, while CACO-2 adhered preferentially to HUVEC compared to HLEC. The breast cancer cell line MCF-7 adheres poorly to both endothelium in contrast to HT1080 and CACO-2.
Figure 3.7 Effect of cancer cell concentration on CACO-2 (A) and HT1080 (B) adhesion on HLECs under static conditions. Cancer cells suspensions were added to the confluent HLEC monolayers with a total volume of 200µl on a 96 well plate at 2.5(●) and 10(●) x10^5 cells/ml, respectively. Cancer cells were allowed to adhere at 37 °C at the indicated time points. Thereafter, non-adherent cancer cells were washed away and the remaining fluorescence per well was measured on a plate reader. N=3. Data points represent the mean (±S.D.) fluorescence intensity.
Figure 3.8 Effect of cancer cell concentration on CACO-2 (A) and HT1080 (B) adhesion on HUVEC under static conditions. Different concentrations of cancer cells suspensions were added to the confluent HUVEC monolayers with a total volume of 200µl on a 96 well plate at 2.5(●) and 10(■) x10^5 cells/ml, respectively. Cancer cells were allowed to adhere at 37 °C at the indicated time points. Thereafter, non-adherent cancer cells were washed away and the remaining fluorescence per well was measured on a plate reader. N=3. Data points represent the mean (±S.D.) fluorescence intensity.
Figure 3.9 Time course of CACO-2, HT-1080 and MCF-7 cell adhesion to the HLEC monolayer under static conditions. Cancer cells were labeled with 1µM CellTracker™ Green CMFDA and added to the confluent HLEC monolayers on a 96 well plate at 2×10^5 cells/well and allowed to adhere at 37 °C at the indicated time points. Thereafter, non-adherent cancer cells were washed away and the remaining fluorescence per well was measured on a plate reader. Data points represent the mean (±S.D.) fluorescence intensity determined from three independent experiments.
Figure 3.10 The fluorescence images of adherent cancer cells on the HLEC monolayer at the indicated time points.

The cancer cells were labeled with 1μM CellTracker™ Green CMFDA and added to the confluent endothelial monolayers on a 96 well plate at 2x10^5 cells/well and allowed to adhere at 37°C at the indicated time points. Thereafter, non-adherent cancer cells were washed away and the remaining cells were photographed under a fluorescence microscope.
Figure 3.11 The fluorescence images of adherent cancer cells on the HUVEC monolayer at the indicated time points. The cancer cells were labeled with 1µM CellTracker™ Green CMFDA and added to the confluent endothelial monolayers on a 96 well plate at 2x10^5 cells/well and allowed to adhere at 37°C at the indicated time points. Thereafter, non-adherent cancer cells were washed away and the remaining cells were photographed under a fluorescence microscope.
3.1.6. Effect of NO and ONOO$^-$ on cancer cell adhesion to unstimulated HLEC under static conditions

Based on our previous findings, we hypothesized that nitrooxidative/oxidative stress would promote cancer cell adhesion. To test this hypothesis, we investigated several NO and ONOO$^-$ modulators on their potential to regulate cancer cell adhesion to the lymphatic endothelium.

3.1.6.1. Effect of SIN-1

Peroxynitrite is a relatively long-lived, highly reactive diffusible molecule and may modulate the signaling function of NO (122), making it a good candidate molecule for mediating NO and O$_2^-$ oxidative damage to cells (123). SIN-1 is known to simultaneously generate equal amounts of NO and O$_2^-$ during decomposition, which interact almost instantly to produce ONOO$^-$ (124). The reaction rate between NO and O$_2^-$ is near diffusion limited (rate constant= 6.7×10$^9$ M$^{-1}$ s$^{-1}$), making SIN-1 an effective ONOO$^-$ donor (125).

Figure 3.12A shows that the incubation with SIN-1 (0.1–3mM) for 1h did not produce any significant change in adhesion of CACO-2 cells. While 0.5mM SIN-1 increased the adhesion of HT1080 cells by 20% (P<0.05).

Figure 3.12B shows that exposure of non-activated HLEC to 3mM SIN-1 for 2h significantly increased the adhesion of CACO-2 and HT1080 by 45% and 60% respectively. This is consistent with a study showing that 3mM SIN-1 most effectively
generate ONOO$^-$ with a rate of roughly 30µM· min$^{-1}$ that is maintained for at least 1 h (126).
Figure 3.12 Effect of SIN-1 on CACO-2 and HT1080 cell adhesion on HLECs under static conditions. HLECs were incubated with various concentrations of SIN-1 for 1h (A) or 2h (B) before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student's t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
3.1.6.2. Effect of SNAP

In the present study, our hypothesis is that NO generation \textit{in vitro} can decrease the adhesion of cancer cells to HLEC and thereby suppress lymphatic metastasis. In order to test this assumption, we investigated the effects of SNAP, a NO donor, on the adhesion of CACO-2 and HT1080 cells to HLEC. The results indicate that the adhesion of CACO-2 and HT1080 cells to HLEC can be reduced by SNAP supplementation (Figure 3.13). The incubation of non-activated HLEC with SNAP (0.1mM) for 30min induced approximately 35% inhibition of HT1080 cell adhesion. A less prominent effect was seen in CACO-2 cells. The incubation with SNAP (0.1–2mM) for 30min reduced CACO-2 adhesion by approximately 25% (Figure 3.13A).

![Graph showing the effect of SNAP on CACO-2 and HT1080 cell adhesion on HLECs under static conditions.](image)

Figure 3.13 Effect of SNAP on CACO-2 and HT1080 cell adhesion on HLECs under static conditions. HLECs were incubated with various concentrations of SNAP for 30 minutes before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05; **P<0.01; ***P<0.001 compared with the control.
3.1.6.3. Effect of VAS2870

As is shown in Figure 3.14A, 0.0001mM VAS2870 reduced CACO-2 cell adhesion by around 20% (P<0.01). The incubation with 0.0025mM VAS2870 for 30min decreased CACO-2 adhesion by approximately 25% (Figure 3.14A). A less prominent effect was seen in HT1080 cells. The incubation of non-activated HLEC with 0.0125mM VAS2870 for 30min decreased HT1080 adhesion by around 13% (Figure 3.14A).

3.1.6.4. Effect of L-arginine

L-arginine is the only substrate for eNOS to produce NO in endothelial cells, therefore it has been widely used in biochemical studies to boost NO production (127). As shown in Figure 3.14B, L-arginine (0.1-1mM) attenuated cell adhesion in CACO-2 cell by 10%, but did not attenuate HT1080 cell adhesion.

3.1.6.5. Effect of PEG-SOD and MnTBAP

To find out if O$_2^-$ and ONOO$^-$ could mediate cancer cell adhesion to endothelial cells, the effects of PEG-SOD (O$_2^-$ scavenger) and MnTBAP (ONOO$^-$ scavenger) were studied in the co-culture. PEG-SOD was found to be effective in suppressing CACO-2 cell adhesion over a wide range of concentrations. 300-500U/ml PEG-SOD significantly decreased CACO-2 cell adhesion by 25% compared to the control (Figure 3.15A). A less prominent effect was seen in the HT1080 cell adhesion (15%, p<0.01). While the addition of MnTBAP to the HLECs failed to produce any significant effect on either CACO-2 or HT1080 adhesion (Figure 3.15B).
Figure 3.14  Effect of VAS2870 (A) and L-arginine (B) on CACO-2 and HT1080 cell adhesion on HLECs under static conditions. HLECs were incubated with various concentrations of VAS2870 and L-arginine for 30 minutes before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
Figure 3.15 Effect of PEG-SOD (A) and MnTBAP (B) on CACO-2 and HT1080 cell adhesion on HLECs under static conditions. HLECs were incubated with various concentrations of PEG-SOD (A) and MnTBAP (B) for 30 minutes before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test: *P<0.05; **P<0.01; ***P<0.001 compared with the control.
3.1.7. Effect of NO and ONOO\(^{-}\) on cancer cell adhesion to TNF-\(\alpha\) stimulated HLEC under static conditions

Activation of endothelial cells by cytokines is a process that must be expected during metastasis \textit{in vivo} (128). Thus, comparative studies in the presence and absence of cytokines appeared necessary. Therefore we pretreated HLECs with TNF-\(\alpha\) (1ng/ml) for 24 h before incubation of the HLECs with other agents.

3.1.7.1. Effect of SIN-1

It is possible that the production of ONOO\(^{-}\) may cause endothelial damage and promote cancer cell/endothelial interaction under inflammation. In order to examine this possibility, the effect of SIN-1 (ONOO\(^{-}\) donor) on the adherence of cancer cells to TNF-\(\alpha\) -treated HLEC was examined.

The effect of SIN-1 on CACO-2 and HT1080 cell adhesion was investigated by incubating TNF-\(\alpha\) stimulated HLECs for 1 h or 2h with various concentrations of SIN-1. Figure 3.16A showed that the presence of SIN-1 for 1h dose-dependently increase the adhesion of both CACO-2 and HT1080 cells to HLECs. 1h incubation with of SIN-1 increased CACO-2 and HT1080 adhesion by 35\% (\(P<0.01\), observed with 3mM SIN-1) and 27\% (\(P<0.01\), observed with 2mM SIN-1) respectively.

However, incubation with SIN-1for 2h attenuated cancer cell adhesion to the endothelial monolayer in a dose dependent manner. Specifically, 1mM SIN-1 reduced HT1080 cell adhesion by 20\%, \(P<0.01\)(Figure 3.16B).
Figure 3.16 Effect of SIN-1 on CACO-2 and HT1080 cell adhesion on TNF-α-stimulated HLECs under static conditions. TNF-α- stimulated HLECs were incubated with various concentrations of SIN-1 for 1h(A) or 2h(B) before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
3.1.7.2. Effect of L-arginine

Treating HLECs with L-arginine for 1h showed a decrease in CACO-2 adhesion (Figure 3.17A), with the decrease being statistically significant at 1mM (23%, p<0.01) compared to the control. Similar results were observed with HT1080 adhesion (30%, p<0.05).

2h incubation showed a similar effect in CACO-2 adhesion (Figure 3.17B). While the inhibitory effect of L-arginine on HT1080 adhesion was diminished after 2h treatment.

3.1.7.3. Effect of SNAP

Pretreatment of HLECs with SNAP reduced CACO-2 cell adhesion over a wide range of concentrations. 0.00013mM SNAP reduced CACO-2 cell adhesion by 15% (P<0.01). Similar results were observed with HT1080 cell adhesion. 0.01mM SNAP reduced HT1080 cell adhesion by around 25% (Figure 3.18). These results suggest that NO has anti-adhesive properties and enhanced NO release was associated with less cancer cell adhesion to the endothelium.

Incubation with SNAP for 2h had a similar effect on CACO-2 adhesion at a low concentration (0.00013mM). However, the inhibitory effect of SNAP at higher concentrations (>0.01mM) was diminished with 2h incubation (Figure 3.18). Similar results were observed with HT1080 cell adhesion.
Figure 3.17 Effect of L-arginine on CACO-2 and HT1080 cell adhesion on TNF-α-stimulated HLECs under static conditions. TNF-α- stimulated HLECs were incubated with various concentrations of L-arginine for 1h(A) or 2h(B) before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
Figure 3.18 Effect of SNAP on CACO-2 and HT1080 cell adhesion on TNF-α-stimulated HLECs under static conditions. TNF-α- stimulated HLECs were incubated with various concentrations of SNAP for 1h (A) or 2h (B) before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test: *P<0.05; **P<0.01; ***P<0.001 compared with the control.
3.1.7.4. Effect of VAS2870

As shown in Figure 3.19A, 1h treatment with VAS2870 decreased CACO-2 cell adhesion by 20% (P<0.05) at 0.01mM, followed by a significant increase at 0.08mM, then a significant decrease in cell adhesion was observed again with 0.3mM VAS2870 (40%, P<0.01). However, 1h incubation with VAS2870 showed no significant effect on adhesion of HT1080 to TNF-α activated HLEC (Figure 3.19A).

Pretreatment of TNF-α-stimulated HLEC with VAS2870 for 2 h had a much more dramatic effect than 1h incubation, with a 20% decrease (p<0.001) in CACO-2 adhesion starting at 0.01mM and a dramatic 60% decrease (p<0.001) at 0.025mM (Figure 3.19B). The pattern observed for HT1080 is different, with an initial 25% increase at 0.025mM VAS2870, and then decrease significantly at higher concentrations. A maximum 40% decrease of HT1080 adherence was observed with 0.3mM VAS2870 (P<0.001).
Figure 3.19 Effect of VAS2870 on CACO-2 and HT1080 cell adhesion on TNF-α-stimulated HLECs under static conditions.

TNF-α- stimulated HLECs were incubated with various concentrations of VAS2870 for 1h (A) or 2h (B) before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
3.1.7.5. Effect of PEG-SOD

We next examined if PEG-SOD had an effect on the adhesion of CACO-2 and HT1080 cells to TNF-α HLEC. The data showed that 1 h treatment with PEG-SOD (800U/ml) reduced the adhesion of CACO-2 by around 25% (P<0.01), compared to the TNF-α-treated HLECs control. 2 h treatment with PEG-SOD produced a similar result in CACO-2 adhesion.

In HT1080 cells, 1h treatment with 800U/ml PEG-SOD reduced HT1080 adhesion by around 25% (P<0.01). However, 2 h treatment with PEG-SOD failed to produce any significant result on the adhesion of HT1080 cells to HLEC (Figure 3.20B).

3.1.7.6. Effect of MnTBAP

The data showed that 1 h treatment with MnTBAP reduced the adhesion of CACO-2 by around 15%-30%, compared to the TNF-α-treated HLECs control. A low concentration (0.0004mM) was able to reduced CACO-2 adhesion by 30% (P<0.01). 2h incubation with MnTBAP produced similar results.

As can be seen in Figure 3.21A, MnTBAP treatment resulted in a dose-dependent decrease of HT1080 adhesion to TNF-α stimulated HLEC. In the presence of 0.04mM MnTBAP for 1h, HT1080 adhesion was decreased by 30% (P<0.01). However, 2h incubation with MnTBAP did not produce a significant effect on the adhesion of HT1080 cells to HLEC (Figure 3.21B).
Figure 3.20 Effect of PEG-SOD on CACO-2 and HT1080 cell adhesion on TNF-α-stimulated HLECs under static conditions. TNF-α-stimulated HLECs were incubated with various concentrations of PEG-SOD for 1h (A) or 2h (B) before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test: *P<0.05; **P<0.01; ***P<0.001 compared with the control.
Figure 3.21 Effect of MnTBAP on CACO-2 and HT1080 cell adhesion on TNF-α-stimulated HLECs under static conditions. TNF-α-stimulated HLECs were incubated with various concentrations of MnTBAP for 1h(A) or 2h(B) before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
3.1.7.7. Effect of L-NAME

When the endothelial cells were pretreated with L-NAME to inhibit NOS activity, a significant decrease in cancer cell adhesion was observed (Figure 3.22). Incubation with L-NAME(0.0005-0.5mM) for 1h significantly reduced CACO-2 cell adhesion by 20-25%. Incubation with L-NAME for 2h has a less prominent effect in CACO-2 adhesion. A 20% decrease (P<0.01) was observed only with 0.005mM L-NAME, while other concentrations of L-NAME showed no significant effect.

Similarly, incubation with L-NAME (0.0005-0.5mM) for 1hour significantly reduced HT1080 cell adhesion by around 20%. However, 2h incubation with L-NAME failed to produce a significant effect on the adhesion of HT1080 cells to HLEC (Figure 3.22B).
Figure 3.22 Effect of L-NAME on CACO-2 and HT1080 cell adhesion on TNF-α-stimulated HLECs under static conditions.

TNF-α-stimulated HLECs were incubated with various concentrations of L-NAME for 1h(A) or 2h(B) before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student's t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
3.1.7.8. Summary

Our data showed that preincubation of HLECs with L-NAME for 30 min did not produce a significant effect on the adhesion of either CACO-2 or HT1080, but the incubation with PEG-SOD (400 U/ml) decreased both CACO-2 and HT1080 adhesion by 20%, as compared to the control (Figure 3.23 and Figure 3.24). Similar results were observed for L-arginine (0.5 mM), VAS2870 (0.005 mM), MnTBAP (0.02 mM) and SNAP (0.5 mM). In contrast, the ONOO⁻ donor, SIN-1 (2 mM) increases CACO-2 adhesion to the HLEC monolayer by 10%.

Preincubation of HLEC with TNF-α (1 ng/ml, 24 h) increased adhesion of all three cancer cell lines compared to adhesion in the control group. In the presence of L-arginine (0.5 mM) for 1 h, the adhesion of both CACO-2 and HT1080 was reduced by 20% (Figure 3.25 and Figure 3.26), compared to the TNF-α-treated HLECs control. Similar results were obtained with SNAP (0.5 mM), PEG-SOD (400 U/ml), MnTBAP (0.2 mM) and L-NAME (0.05 mM). By contrast, treatment of the HLEC with SIN-1 (2 mM) increased CACO-2 and HT1080 adhesion by 30% and 20% respectively.

Similarly, treatment of the TNF-α-treated HLECs with L-arginine (0.5 mM), SNAP (0.5 mM), PEG-SOD (400 U/ml) and MnTBAP (0.2 mM) for 2 h reduced the cancer cell adhesion by 20%. But 2 h incubation with L-NAME did not interfere the adhesion of HT1080 to the HLECs (Figure 3.28). We also found that the NADPH oxidase inhibitor, VAS2870 interfered the adhesion of cancer cells to HLECs in a dose-dependent manner. 0.3 mM VAS2870 reduced CACO-2 adhesion by almost 50% (Figure 3.27).
Figure 3.23 Effect of NO and ONOO\(^-\) on CACO-2 cell adhesion on HLECs under static conditions. SIN-1(2 mM), L-arginine(0.5 mM), SNAP (0.5 mM), VAS2870(0.005 mM), PEG-SOD(400 U/ml), MnTBAP(0.02 mM) and L-NAME (0.5 mM) was added to HLECs for 30 min before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
Figure 3.24 Effect of NO and ONOO⁻ on HT1080 cell adhesion on HLECs under static conditions.
SIN-1(0.1mM), L-arginine(0.5mM), SNAP (0.5mM), VAS2870(0.0025mM), PEG-SOD(500U/ml), MnTBAP(0.004mM) and L-NAME (0.005mM) was added to HLECs for 30min before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
Figure 3.25 Effect of NO and ONOO· on CACO-2 cell adhesion on TNF-α- stimulated HLECs under static conditions.
SIN-1(3mM), L-arginine(0.5mM), SNAP (0.1mM), VAS2870(0.3mM), PEG-SOD(400U/ml), MnTBAP(0.0004mM) and L-NAME (0.005mM) was added to TNF-α-pretreated HLECs (1ng/ml) for 1h before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
Figure 3.26 Effect of NO and ONOO⁻ on HT1080 cell adhesion on TNF-α- stimulated HLECs under static conditions.

SIN-1(2mM), L-arginine(0.5mM), SNAP (0.1mM), VAS2870(0.01mM), PEG-SOD(800U/ml), MnTBAP(0.04mM) and L-NAME (0.05mM) was added to TNF-α- pretreated HLECs (1ng/ml) for 1h before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
Figure 3.27 Effect of NO and ONOO⁻ on CACO-2 cell adhesion on TNF-α- stimulated HLECs under static conditions.
SIN-1(3mM), L-arginine(0.5mM), SNAP (130nM), VAS2870(0.3mM), PEG-SOD(600U/ml), MnTBAP(0.02mM) and L-NAME (0.005mM) was added to TNF-α-pretreated HLECs (1ng/ml) for 2h before the static adhesion assay. Results are calculated as percentage of the control group. Data shown are the means ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
3.1.8. Effect of NO and ONOO⁻ on ICAM-1 and VCAM-1 expression on unstimulated HLEC

Having demonstrated the effect of NO and ONOO⁻ exposure on cancer cell adhesion, we next examined the underlying mechanism, focusing on the expression level of ICAM-1 and VCAM-1 which is known to play important roles in cell–cell interaction.
3.1.8.1. Effect of VAS2870

Because ICAM-1 and VCAM-1 has been shown to play an important role in the adhesion of cancer cells to endothelial cells and therefore in metastasis, we sought to examine whether VAS2870 affects the expression of these adhesion molecules in HLECs. Toward this aim, cells were treated with various concentrations of VAS2870 for 30 minutes and the protein level of the ICAM-1 and VCAM-1 was determined by ELISA. As is shown in Figure 3.29A, the level of ICAM-1 and VCAM-1 protein decreased in concentration-dependent manner in VAS2870-treated HLECs. The expression of VCAM-1 was significantly down-regulated by approximately 40% by treatment with VAS2870 (0.0025-0.025mM). The inhibitory effect of VAS2870 on VCAM-1 expression was less prominent. A 25% and 50% decrease in VCAM-1 expression was observed with 0.0125mM and 0.025mM VAS2870 respectively. Taken together, our results suggest that VAS2870 exerts an inhibitory effect on the adhesion of cancer cells to non-stimulated HLEC and that this effect is associated with a down-regulation of ICAM-1 and VCAM-1 protein expression.

3.1.8.2. Effect of MnTBAP, PEG-SOD and SNAP

Cell surface expression of ICAM-1 and VCAM-1 on HLEC was evaluated 30 minutes after incubation with MnTBAP, and the results were compared to untreated control HLECs. As shown in Figure 3.29B, no significant change was observed in ICAM-1 protein content of endothelial cells treated with MnTBAP at concentrations lower than 0.004mM. However, a 10% decrease in ICAM-1 expression was observed
with MnTBAP at concentrations range from 0.02mM to 0.06mM. Similar results were observed with VCAM-1 expression. A 10% decrease in VCAM-1 expression was observed over the range between 0.04 and 0.06mM MnTBAP. Addition of PEG-SOD (200-800 U/ml) has no significant effect on ICAM-1 or VCAM-1 expression (Figure 3.30A). While a 20% decrease in ICAM-1 expression was observed with 1000 U/ml PEG-SOD (P<0.01). The presence of SNAP has no significant effect on VCAM-1 expression (Figure 3.30B), while SNAP mildly reduced ICAM-1 molecule at concentrations range from 0.1 to 2mM.
Figure 3.29 Effects of VAS2870(A) and MnTBAP(B) on ICAM-1 and VCAM-1 expression in HLECs. HLECs were exposed to different concentrations of VAS2870(A) and MnTBAP(B) for 30 minutes. Adhesion molecule expression was determined by cellular ELISA (OD measured at 450nm). Results are expressed as percentage of the control. N=3. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
Figure 3.30 Effects of PEG-SOD(A) and SNAP(B) on ICAM-1 and VCAM-1 expression in HLECs.

HLECs were exposed to different concentrations of PEG-SOD(A) and SNAP(B) for 30 minutes. Adhesion molecule expression was determined by cellular ELISA (OD measured at 450nm). Results are expressed as percentage of the control. N=3. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
3.1.9. Effect of NO and ONOO\(^{-}\) on ICAM-1 and VCAM-1 expression on TNF-\(\alpha\) stimulated HLEC

3.1.9.1. Effect of SIN-1 and VAS2870

Addition of SIN-1 (0.1-3mM) significantly increased TNF-\(\alpha\)-stimulated VCAM-1 expression by 10% (Figure 3.31A). However, no change was observed in ICAM-1 expression.

The presence of VAS2870 dose-dependently reduced the level of ICAM-1 and VCAM-1 expression, with TNF-\(\alpha\) stimulated HLECs exposed to 0.3mM VAS2870 expressing the least amount of ICAM-1 (10%, \(P<0.001\)) and VCAM-1 (35%, \(P<0.001\)) (Figure 3.31B).

3.1.9.2. Effect of MnTBAP

The treatment with MnTBAP for 1 h had no effects on the expression of ICAM-1 at concentrations lower than 0.04mM. A mild decrease (5%, \(P<0.01\)) in ICAM-1 expression was observed with 0.06 and 0.08mM MnTBAP. A 10% decrease was observed in VCAM-1 expression with MnTBAP (0.0004-0.06mM). VCAM-1 expression was most significantly decreased (30%, \(P<0.001\)) with 0.08mM MnTBAP. These data suggest that at low concentrations (lower than 0.06mM), the inhibitory effect of MnTBAP on cancer cell adhesion may be due to the decrease of ICAM-1 level, while at high concentrations both ICAM-1 and VCAM-1 expression is responsible for inhibitory effect of MnTBAP on cancer cell adhesion.
3.1.9.3. Effect of PEG-SOD

We next investigated the effect of PEG-SOD on the inhibition of ICAM-1 and VCAM-1 expression. As shown in Figure 3.32B, the addition of PEG-SOD (200-1000U/ml) to the culture medium did not affect the inhibition of ICAM-1 expression, suggesting that ICAM-1 expression may not play a major role in mediating the inhibitory effect of PEG-SOD on cancer cell adhesion. However, 400 and 800 U/ml PEG-SOD significantly reduce VCAM-1 expression by 10% (P<0.05), indicating that inhibition of VCAM-1 expression may be responsible for the effect of PEG-SOD on cancer cell adhesion.

3.1.9.4. Effect of L-arginine and SNAP

L-arginine had no noticeable effects on the expression of ICAM-1 and VCAM-1 (Figure 3.33A). This is consistent with a study showing that short term L-arginine supplementation does not change the expression of adhesion molecules on endothelial cells (127). Therefore other adhesion molecule may be responsible for the inhibitory effect of L-arginine on cancer cell adhesion.

To examine the possible involvement of NO in the expression of both ICAM-1 and VCAM-1 expression, we treated HLEC with a NO donor, SNAP. The treatment with SNAP (2mM) caused a slightly decrease (5%, P<0.01) in the levels of ICAM-1 in TNF-α stimulated HLECs (Figure 3.33B) but had no effect on VCAM-1 expression.
Figure 3.31 Effects of SIN-1(A) and VAS2870(B) on TNF-α-induced ICAM-1 and VCAM-1 expression in HLECs.

After 24-hour stimulation with TNF-α (1ng/ml), HLECs were exposed to different concentrations of SIN-1(A) and VAS2870 (B) for 1h. Adhesion molecule expression was determined by cellular ELISA (OD measured at 450nm). Results are expressed as percentage of the control. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control. N=3.
Figure 3.32 Effects of MnTBAP(A) and PEG-SOD(B) on TNF-α-induced ICAM-1 and VCAM-1 expression in HLECs.

After 24-hour stimulation with TNF-α (1ng/ml), HLECs were exposed to different concentrations of MnTBAP(A) and PEG-SOD(B) for 1h. Adhesion molecule expression was determined by cellular ELISA (OD measured at 450nm). Results are expressed as percentage of the control. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control. N=3.
Figure 3.33 Effects of L-arginine (A) and SNAP (B) on TNF-α-induced ICAM-1 and VCAM-1 expression in HLECs.
After 24-hour stimulation with TNF-α (1ng/ml), HLECs were exposed to different concentrations of L-arginine (A) and SNAP (B) for 1h. Adhesion molecule expression was determined by cellular ELISA (OD measured at 450nm). Results are expressed as percentage of the control. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control. N=3.
3.1.9.5. Summary

Figure 3.29A shows that treatment with VAS2870 decreased the levels of ICAM-1 and VCAM-1 on HLECs in a dose dependent manner. Maximal inhibition of ICAM-1 and VCAM-1 was achieved with 0.025mM VAS2870 (45 and 47% inhibition, respectively). Treatment with MnTBAP (0.02, 0.04mM) reduced ICAM-1 and VCAM-1 expression by 10% (Figure 3.29B). Addition of SNAP reduced ICAM-1 expression by approximately 5%, although it showed no effect on VCAM-1 expression (Figure 3.30B). In contrast, treatment with PEG-SOD (400U/ml) reduced VCAM-1 expression by approximately 5%, but has no effect on VCAM-1 expression (Figure 3.30A).

Because TNF-α increased both ICAM-1 and VCAM-1 expression at 24h significantly, we evaluated whether NO inhibits TNF-α induction of cell adhesion molecules in the following experiments (129). We found that in HLECs stimulated with TNF-α, SNAP did not influence the expression of ICAM-1 or VCAM-1 over a range of concentrations (Figure 3.33B), suggesting that the inhibition of cancer cell adhesion by SNAP is independent of inhibition of ICAM-1 or VCAM-1 expression and there may be other adhesion molecules responsible for the anti-adhesive effect of SNAP. As exogenous NO failed to inhibit TNFα-stimulated expression of adhesion molecules, we next looked at whether endogenous NO could prove more effective. We found that treatment with L-arginine (0.5mM) did not affect TNF-α-induced ICAM-1 expression, but mildly inhibited TNF-α -induced VCAM-1 expression by 5% (Figure 3.33A).Because nitric oxide can combine with superoxide to form reactive peroxynitrite, which may alter the expression of membrane proteins, we investigated the effect of superoxide radical scavengers on the
inhibition of ICAM-1 and VCAM-1 expression by VAS2870, PEG-SOD and MnTBAP. As shown in Figure 3.31B, addition of VAS2870 significantly suppressed TNF-α-induced ICAM-1 and VCAM-1 expression in a dose-dependent manner. The inhibitory effect of VAS2870 was more prominent on the expression of VCAM-1. 0.3mM VAS2870 significantly reduced TNF-a-induced VCAM-1 more effectively compared to that of ICAM-1 (35 vs. 10% inhibition, respectively). Incubation with MnTBAP (0.0004, 0.04mM) and PEG-SOD (400, 800U/ml) did not produce a significant effect on TNF-α-stimulated ICAM-1 expression, but decrease the expression of VCAM-1 by around 10% (Figure 3.32), indicating that down-regulation of VCAM-1 may be the responsible for the anti-adhesive effect for these agents. We next examined the effects of SIN-1 on TNF-α-induced ICAM-1 and VCAM-1 expression. As shown in Figure 3.31A, the incubation with SIN-1 (3mM) increased TNF-α-stimulated ICAM-1 and VCAM-1 expression by approximately 10%. These results suggest that NO could prevent the adhesion of cancer cells through the inhibition of ICAM-1 and VCAM-1 expression on HLEC.

3.2. Flow adhesion

3.2.1. Effect of TNF-α on cancer cell adhesion under flow condition

Figure 3.34 is a digitized image of HT1080 cell adhesion on HLEC after 10 min of perfusion at 0.5 dyne/cm². Our data showed that only a few CACO-2 or HT1080 cells can adhere to non-stimulated HLEC under flow conditions. However, increased amount of adhesion was observed when HLEC were pre-incubated with TNF-α. There was a steady increase in the adhesion of cancer cells with increased concentration of TNF-α.
between 1ng/ml and 50ng/ml. However, the number of adherent cells reached a plateau when the TNF-α concentration is within the range of 50ng/ml to 500ng/ml. Therefore, HLECs were pre-incubated with 50ng/ml TNF-α for 24h for all the following experiments under flow.
Figure 3.34 Digitized image of CACO-2(A), HT1080 (B) and MCF-7 (C) cell adhesion on TNF-α stimulated HLEC under flow condition.

Photo was taken under microscope at 10-min perfusion at shear stress of 0.5dyne/cm². HLECs were preincubated with TNF-α 50ng/ml for 24 hours before the adhesion assay.
Figure 3.35 Effect of TNF-α concentration on the adhesion of CACO-2 cells to the HLEC monolayer.
HLEC monolayers were incubated with different concentrations of TNF-α for 24 hours. Then CACO-2 cell suspensions (1x10⁶ cells/ml) were perfused over HLEC monolayers in the parallel plate flow chamber at a physiologic shear stress of 0.5 dyne/cm². After 10 min of perfusion, the number of adherent cells from 3 different microscopic fields was counted. Data are expressed as the mean ± S.D.

3.2.2. NO and ONOO⁻ release from TNF-α stimulated HLEC during tumor cell adhesion under flow conditions

Since static assays do not take into account the effect of wall shear rates related to blood flow and may not truly reflect in vivo events, we developed a flow chamber to further monitor the NO and ONOO⁻ release from HLECs under flow conditions. The maximum NO concentration induced by CACO-2 cells almost doubled
that observed for the control after incubation with L-arginine (0.5mM). We assume the progressive improvement in NO release with L-arginine treatment is related to reversal of eNOS uncoupling, then we would expect to see a corresponding decrease in ONOO\(^-\) production. This was indeed the case as shown in Figure 3.36B. As a result, the [NO]/[ONOO\(^-\)] ratio was significantly shifted toward the increase in bioavailability of NO to 1.26±0.18. The decrease in O\(_2^-\) had a significant influence on both the level of NO and ONOO\(^-\) concentration, as reflected by treatment with PEG-SOD and MnTBAP. In the presence of PEG-SOD (200 U/ml) or MnTBAP (0.04mM), a significant reduction in ONOO\(^-\) concentration with concomitant increase in the NO level was observed for both CACO-2 and HT1080 stimulation. The relative ratio of NO to ONOO\(^-\) release was improved by nearly 2-fold in the PEG-SOD group compared to the control group (Figure 3.37). To test a contribution of the NAD(P)H– generated O\(_2^-\) to the overall formation of ONOO\(^-\) and diminished bioavailable NO, we measured NO and ONOO\(^-\) in the presence of VAS2870, a specific NAD(P)H oxidase inhibitor. A similar effect of the increase in NO and proportional decrease in ONOO\(^-\) was noticed in the presence of the NADPH oxidase inhibitor, VAS2870 (0.01mM). The Inhibition of the NADPH oxidase increases NO concentration by 20-30% and decreased ONOO\(^-\) by about 30% after stimulation with CACO-2 or HT1080 cells (Figure 3.36). This indicates that about 20-30% of NO reacted with O\(_2^-\) produced by NADPH oxidase. We also measured the effects of SNAP (0.5mM) on HLECs NO and ONOO\(^-\) release. Here again, we see a significant improvement in NO generation. This improvement was evident in CACO-2 but with the most significant increase in HT1080-stimulated NO release which was increased by more than 20%, while
HT1080-stimulated ONOO$^{-}$ concentration was decreased by approximately 30% (Figure 3.36A and B). We also confirmed that the release of NO and ONOO$^{-}$ was related to eNOS activation, because inhibition of eNOS by L-NAME blocked significantly HT1080-stimulated release of both NO and ONOO$^{-}$. 
Figure 3.36 Maximal NO(A) and ONOO⁻(B) concentration release from HLECs under flow conditions.

After 24-hour stimulation with TNF-α (50ng/ml), HLECs were incubated with MnTBAP (0.04mM), SNAP (0.5mM), PEG-SOD(200U/ml), VAS2870 (0.01mM), L-arginine (0.5mM),SIN-1 (1mM) and L-NAME (1mM) for 1 hour. Then cancer cell suspensions (1x10⁶ cells/ml) were perfused over HLEC monolayers at a physiologic shear stress of 0.5 dyne/cm². Values are mean ± S.D. (N=4-5). Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
Figure 3.37 The [NO]/[ONOO−] ratio calculated from the maximal NO and ONOO− concentration release from HLECs under flow condition. N=4-5. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.
Figure 3.38 Effect of NO and ONOO$^-$ on cancer cells adhesion on HLECs under flow conditions.

After 24-hour stimulation with TNF-α (50ng/ml), HLECs were incubated with MnTBAP (0.04mM), SNAP (0.5mM), PEG-SOD(200U/ml), VAS2870 (0.01mM), L-arginine (0.5mM),SIN-1 (1mM) and L-NAME (1mM) for 1 hour. Then CACO-2 and HT1080 cell suspensions (1x10$^6$cells/ml) were perfused over HLEC monolayers at a shear stress of 0.5 dyne/cm$^2$. After 10 min of perfusion, the number of adherent cells from 3 different microscopic fields was counted. Data are expressed as the mean ± S.D. from three independent experiments. Two-tailed Student’s t-test:*P<0.05;**P<0.01;***P<0.001 compared with the control.

3.2.3. Effect of NO or ONOO$^-$ on cancer cell adhesion to TNF-α stimulated HLEC under flow condition

However, endothelial cells are constantly exposed to shear stress and static assays poorly simulate adhesion that occurs in blood, or lymph vessels, under shear stress (130). Lymphatic flow is another important factor in regulating tumor cell attachment to the lymphatic endothelium. Therefore, we examine cancer cell adhesion to TNF-α stimulated HLECs under flow conditions. The use of a flow chamber enables us to produce intermittent flow in vitro that mimics physiological flow in the lymphatics.
Our results suggest that CACO-2 and HT1080 adhesion was significantly augmented by pre-stimulation of HLEC with 50ng/ml TNF-α for 24 hrs. However, MCF-7 cells are unlikely to establish adhesive interactions with the lymphatic endothelium when perfused at normal physiological shear rates. Our results also showed that if flow is transiently reduced, tumor cells can form strong adhesive contacts that allow the cells to remain adherent even in the face of substantial subsequent increases in shear stress. These findings support the hypothesis that the interaction of circulating tumor cells with the lymphatic endothelium may be enhanced at sites of inflammation or when shear rates are low.

We next examined the effect of several NO and ONOO⁻ modulators on the adhesion under flow condition. Our data showed that treatment of TNF-α-treated HLECs with SIN-1(1mM) for 1hr did not produce a significant effect on the adhesion of either CACO-2 or HT1080, but the incubation with the nitric oxide donor, SNAP (0.5mM) decreased CACO-2 and HT1080 adhesion by 40% and 50% respectively (Figure 3.38). Similarly, treatment with PEG-SOD (200U/ml) also significantly inhibited adherence of both cancer cell lines by almost 50%. A similar pattern was observed in the presence of MnTBAP (0.04mM) and VAS2870 (0.01mM). This result is best explained by the prevention of ONOO⁻ formation via O₂⁻ scavenging with these agents.
3.2.4. Effect of shear stress on cancer cell adhesion to TNF-α stimulated HLEC under flow condition

The HLEC monolayers were pretreated with TNF-α (50ng/ml) 24h before the flow adhesion assay. Our data showed that both CACO-2 and HT1080 adhesion is shear stress-dependent and time-dependent under flow condition. The amount of adherent cells increased with time for both CACO-2 and HT1080 (Figure 3.39). Our data also showed that slight changes in wall shear stress can cause large changes in cancer cell adhesion to TNF-α-stimulated HLEC. Increasing the wall shear stress from 0.1 to 0.5 dynes/cm² reduced the number of adherent CACO-2 cells by a factor of two on TNF-α-treated endothelium. Further increase the wall shear stress to 0.8dynes/cm² decreased the absolute number of adherent CACO-2 dramatically (Figure 3.39A). Similar results were observed with HT1080 cell adhesion. These results suggest that NO may be important in regulating cancer cell-lymphatic endothelial cell interaction by regulating vessel dilation and local lymph flow.
Figure 3.39 Effect of shear stress on CACO-2 (A) and HT1080 (B) cell adhesion on HLECs under flow conditions. Cancer cell suspensions (1x10^6 cells/ml) were perfused over TNF-α-stimulated HLEC monolayers in the parallel plate flow chamber at different shear stresses of 0.1 (●), 0.5 (■) and 0.8 (▲) dyne/cm^2, respectively and for 10-min perfusion periods. N=3.
4. Discussion

4.1. Cancer cells induced an imbalance of [NO]/[ONOO\(^-\)] in the endothelium

Here we describe for the first time the role of [NO]/[ONOO\(^-\)] balance/imbalance in the controlling of cancer cell adhesion to the lymphatic endothelium. A ratio of cytoprotective NO concentration to cytotoxic ONOO\(^-\) concentration (nitroxidative stress) was used as an indicator of functional/dysfunctional endothelial cells. A high level of [NO]/[ONOO\(^-\)] ratio (>2.0) indicates high level of bioavailable NO and normal endothelial function, while a low ratio (<1.0) is associated with dysfunctional endothelium and high nitroxidative/oxidative stress (131).

Using chemical nanosensors, we monitored real-time NO and ONOO\(^-\) release from HLECs following cancer cell stimulation. Our data showed that there was a linear relationship between NO generation and cancer cell concentration in the range between 0.625x10\(^5\) and 5x10\(^5\) cells/ml. NO production decreased significantly when the concentration of cancer cells reached a threshold value, which is 5x10\(^5\) cells/ml, while ONOO\(^-\) production increased with increased cancer cell concentration. As a result, high concentrations of cancer cells, particularly HT1080 cancer cells, unfavorably shifted the ratio of [NO]/[ONOO\(^-\)] to low levels in HLECs and significantly increased the adhesion of tumor cells to the endothelium. Similar results were obtained with HUVECs.

We suggest that the endothelium undergoes a profound degree of cellular dysfunction within minutes (2 to 3 min) in the presence of cancer cells. Most likely the collision of cancer cells with the endothelium stimulates a high initial NO production in endothelial cells, leading to depletion of enzymatic substrates and cofactors (eg, L-
arginine, O₂, or BH4, NADPH, FAD) (132) and, eventually, to uncoupling of eNOS. The uncoupled eNOS can generate large amounts of ROS such as O₂⁻ and ONOO⁻ (133), resulting in an acute increase in oxidative stress that exceeds the cellular defense capacity. O₂⁻ and NO can undergo a rapid diffusion-controlled reaction to form cytotoxic ONOO⁻. Also, ONOO⁻ can hinder NO formation through oxidization of the eNOS cofactor BH4 (134), leading to an overall decrease in the [NO]/[ONOO⁻] ratio. As a result, the beneficial effect of NO can be limited and the generation of ONOO⁻ may initiate a cascade of events leading eventually to endothelial dysfunction (135). Furthermore, ONOO⁻, which is a very strong oxidant, can undergo cleavage to form additional highly oxidative species such as NO₂ and OH⁻ (95,96), leading to nitrooxidative stress which may increase cancer cell adhesion to the endothelium, and consequently, to metastasis. It is our important observation that the increased adhesion of cancer cells occurred at low [NO]/[ONOO⁻] ratio.

Our results strongly suggested that cancer cells induced high oxidative/nitrooxidative stress to endothelial cells, followed by low [NO]/[ONOO⁻] ratio, leading to endothelial dysfunction which may promote cancer cell adhesion.

4.2. The imbalance of [NO]/[ONOO⁻] promotes cancer cell adhesion to HLEC and HUVEC

It appears from this study that maintaining a favorable balance of [NO]/[ONOO⁻] (higher than 1.0) is crucial in preventing cancer cell adhesion to the endothelium. The significant decrease of [NO]/[ONOO⁻] ratio resulted in a marked degree of cancer cell adhesion.
adhesion to HLEC after 10 min (Figure 3.10). A rate of [NO]/[ONOO•], and the adhesion of cancer cells to HLEC depends on the number of cancer cells, type of cancer cells, time of exposure and the flow of medium. Our results are consistent with a previous study in our lab showing that NO inhibits tumor cell adhesion while ONOO• promotes tumor cell adhesion to HUVECs (119).

It has been shown that NO is cytoprotective and suppresses cell adhesion. For example, NO production was shown to decrease the adhesion of human colorectal cancer cells to HUVECs via down-regulation of VCAM-1 expression (42). Similarly, reduction of the NO level by adding the NOS inhibitors cause increased leukocyte–endothelial interactions through the expression of P-selectin, ICAM-1 and VCAM-1 in normal vessels (62). Furthermore, accumulating evidence suggests that increased ONOO• formation can promote cell-cell interaction. For instance, the addition of ONOO• resulted in increased neutrophil adhesion to human coronary artery endothelial cells (93). Another study showed that ONOO• increased endothelial VCAM-1, P-selectin, and E-selectin expression, which may enhance neutrophil-endothelial interaction (92). However, our findings clearly suggest that the most important initial factor which triggers cancer cell adhesion is not an abnormal concentration of NO or ONOO• but the ratio of the concentration of these two molecules.

Although the mechanisms underlying cancer cell-lymphatic endothelial cell interaction are likely multifactorial, it is important to note that decreased [NO]/[ONOO•] ratio associated with oxidative stress and dysfunctional endothelium markedly contributes to this phenomenon. Initial studies suggested that NO may regulate cell
adhesiveness by affecting cellular oxidative stress (59). Indeed, cell adhesion molecules are found to be closely associated with the cellular redox state (54). Studies have shown that NO and oxidative stress regulated VCAM-1 gene expression possibly through the reduction-oxidation sensitive NF-κB (57). In another study, oxidative stress enhanced monocyte adhesion to HUVEC through induction of the gene expression of ICAM-1, E-selectin and MCP-1(58). Similarly, oxidative stress was found to regulate VCAM-1 expression through specific reduction-oxidation sensitive transcriptional factors such as NF-κB (57). ROS can activate redox-sensitive transcription factors such as NF-κB that regulate the expression of endothelial cell adhesion molecules within the microvasculature (56). In our study, we suggest that at low [NO]/[ONOO\textsuperscript{-}] ratio, the oxidative stress may activate endothelial cell adhesion molecules possibly through the reduction-oxidation sensitive NF-κB, which triggers tumor cell adhesion. The oxidative stress (indicated by the imbalance of [NO]/[ONOO\textsuperscript{-}]) can potentially activate NF-κB, resulting in increased ICAM-1 and VCAM-1 expression on HLECs.

4.3. NO supplementation suppresses cancer cell adhesion to HLEC

Based on our previous findings, we hypothesized that nitrooxidative/oxidative stress would promote cancer cell adhesion to HLEC. To test this hypothesis, we investigated several NO and ONOO\textsuperscript{-} modulators on their potential to regulate cancer cell adhesion to the lymphatic endothelium. Our data showed that preincubation of HLECs with L-NAME did not produce a significant effect on the adhesion of either CACO-2 or HT1080, but the incubation with PEG-SOD (400U/ml) decreased both CACO-2 and
HT1080 adhesion by 20%, compared to the control. PEG-SOD dismutates $O_2^-$ into $H_2O_2$ and eliminates the toxic effects of $O_2^-$, thereby reducing ONOO$^-$ production. We observed a similar effect with VAS2870, MnTBAP and SNAP treatment and, to a lesser extent, L-arginine supplementation. All these agents, including PEG-SOD, VAS2870, MnTBAP, SNAP and L-arginine, are known to promote the production of bioavailable NO. Therefore we suggest NO supplementation is effective in suppressing cancer cell adhesion. High levels of NO have been shown to decrease neutrophil adhesion to postcapillary venular endothelium (64) and the addition of NOS inhibitors increased leukocyte–endothelial interactions through the expression of platelet selectin (P-selectin), ICAM-1 and VCAM-1 in normal vessels (62). In another study, it was demonstrated that S-nitrosocaptopril, a NO donor, decreased the adhesion of human colorectal cancer cells to HUVECs via down-regulation of VCAM-1 expression (42).

Incubation with SIN-1 for 30min increased CACO-2 adhesion but decreased HT1080 cell adhesion to the HLEC. SIN-1 simultaneously generates NO and $O_2^-$ under physiological conditions. Studies have shown that SIN-1 behaves more like a NO donor than a ONOO$^-$ donor at relatively low $O_2$ levels (137). Our data showed that HT1080 cells induced a high level of oxidative stress to HLECs under static adhesion, which may be associated with low $O_2$ levels. Therefore SIN-1 may decreased HT1080 adhesion by producing cytoprotective NO instead of ONOO$^-$. Incubation with SIN-1 (3mM) for 2h increases CACO-2 and HT1080 cell adhesion to the HLEC monolayer by 45% and 60% respectively. Studies have shown that 3mM SIN-1 produce roughly 30µM·min$^{-1}$ ONOO$^-$ that is maintained for at least 1h (123). Therefore chronic exposure to SIN-1 may
increase cancer cell adhesion by increasing ONOO$^-$ production and inducing endothelial oxidative stress.

These findings support our hypothesis linking the imbalance of [NO]/[ONOO$^-$] in promoting cancer cell adhesion to the lymphatic endothelium. These observations also emphasize the important role of enhanced NO bioavailability and decreased production of cytotoxic ONOO$^-$ in the process of endothelial dysfunction. Together, enhanced bioavailable NO production may suppress lymphatic metastasis by decreasing attachment of cancer cells to HLEC, while aberrant \textit{in vitro} production of ONOO$^-$ may contribute to this process.

4.4. VAS2870 most effectively suppresses cancer cell adhesion

Activation of endothelial cells by cytokines is a process that is expected during metastasis \textit{in vivo} (128). Therefore we examined cancer cell adhesion in the presence of TNF-$\alpha$. Preincubation of HLEC with TNF-$\alpha$ increased the adhesion of all three cancer cell lines. In the presence of L-arginine, the adhesion of both CACO-2 and HT1080 was reduced significantly, compared to the TNF-$\alpha$-treated HLECs control. Similar results were obtained with SNAP, PEG-SOD, MnTBAP, VAS2870 and L-NAME. Conversely, the incubation with SIN-1 for 1h increases CACO-2 and HT1080 adhesion by 30% and 20% respectively.

Similarly, treatment of the TNF-$\alpha$-treated HLECs with L-arginine, SNAP, PEG-SOD, MnTBAP and L-NAME for 2h reduced CACO-2 and HT1080 cell adhesion by 20%. Specifically, incubation with VAS2870 for 2h induced a concentration-dependent
reduction in the adhesion of both CACO-2 and HT1080 cells. CACO-2 cells were more susceptible to the effect of VAS2870 (maximal inhibition about 60% between 0.15 and 0.3mM) than HT1080 cells (maximal inhibition of 40% at about 0.15mM). This effect of VAS2870 appeared to be related to ICAM-1 and VCAM-1 expression on HLEC. Incubation with SIN-1 (3mM) for 2h increased CACO-2 adhesion by approximately 10% while it failed to produce any significant change in HT1080 cell adhesion.

In summary, SIN-1 was more effective in promoting cancer cell adhesion to non-stimulated HLEC than to TNF-α-stimulated HLEC. Furthermore, the promoting effect of SIN-1 on cancer cell adhesion to TNF-α-stimulated HLEC decreased with increased incubation time. Therefore, chronic exposure to ONOO− may be more detrimental under normal physiological conditions than under inflammatory conditions. This may be explained by the fact that ROS production induced by inflammatory reactions is the major cause of endothelial damage under chronic inflammation, thus the contribution of exogenous ONOO− to endothelial injury is less prominent compared to that induced by cytokines. By contrast, VAS2870 was more effective in suppressing cancer cell adhesion to TNF-α-stimulated HLEC than to non-stimulated HLEC, and the inhibitory effect of VAS2870 on cancer cell adhesion to TNF-α-stimulated HLEC increased with increased incubation time. Studies have shown that NADPH oxidase is one of the most important sources of ROS production during inflammation (138). Therefore, the incubation with VAS2870 may reduce most of ROS production through inhibition of NADPH oxidase, thereby reducing endothelial oxidative stress and cancer cell adhesion during inflammation.
4.5. Agents that reverse eNOS uncoupling suppress cancer cell adhesion to HLEC

Our finding that agents which reduce cellular sources of oxidative and nitrooxidative stress is effective in suppressing cancer cell adhesion to TNF-α stimulated HLEC further support a causal relationship between the [NO]/[ONOO\(^-\)] ratio and metastasis. The observation that PEG-SOD, VAS2870 and MnTBAP is effective in reducing cancer cell adhesion under flow conditions suggests that any scavenger of O\(_2^\cdot\) or ONOO\(^-\) would attenuate cancer cell-lymphatic endothelial cell interaction. The major sources of O\(_2^\cdot\) in endothelial cells includes NAD(P)H oxidase, mitochondria, and nitric oxide synthase (48). PEG-SOD and VAS2870 reduce the production of ONOO\(^-\) in favor of the formation of NO via O\(_2^\cdot\) scavenging. Particularly, VAS2870 increases NO bioavailability through the inhibition of NAD(P)H oxidase, resulting in depletion of intracellular ROS production including O\(_2^\cdot\) (139). The present study shows a highly favorable NO release from HLECs after treatment with PEG-SOD, MnTBAP and VAS2870. This effect is probably due to the concomitant lowering of O\(_2^\cdot\) concentration. Hence, the function and life time of NO is prolonged and the endothelial cells perform efficiently with negligible formation of cytotoxic ONOO\(^-\).

Our findings also demonstrate that high concentrations of NO generated either endogenously with L-arginine or exogenously with SNAP decreased cancer cell adhesion to the lymphatic endothelium. L-arginine, the substrate for NO synthesis, restored eNOS coupling, reversed endothelial dysfunction and increased the NO/ONOO\(^-\) ratio, resulting in significant decrease in tumor cell adhesion. The inhibitory effect of L-arginine was apparent in HT1080 cell adhesion, and was very significantly in CACO-2 cell adhesion to
HLECs. The favorable kinetics of NO production in the presence of SNAP is important in maintaining a high level of NO concentration between the endothelium and cancer cells, which provides efficient protection against cancer cell adhesion to the endothelial monolayer.

L-NAME inhibits eNOS expression, and one can expect that L-NAME can promote cancer cell adhesion by reducing NO production. Surprisingly, our results showed that L-NAME treatment cause a significant decrease in cancer cell adhesion to the TNF-α-activated lymphatic endothelium. This beneficial effect of L-NAME can be explained by the fact that L-NAME prevents also the uncoupling of eNOS, reducing the levels of both O$_2^-$ and ONOO$^-$. 

Surprisingly, SIN-1 failed to produce significant increase in either CACO-2 or HT1080 cell adhesion to TNF-α-stimulated HLECs under flow condition. It may be due to that the concentration of SIN-1(1mM) we used under flow conditions is not sufficient to produce cytotoxic amount of ONOO$^-$, as studies have shown that SIN-1 at 3mM most effectively produced ONOO$^-$(123).

The measurement of NO and ONOO$^-$ release from HLECs further reveal that the adhesion of cancer cells is favored by a low [NO]/[ONOO$^-$] ratio or high oxidative/nitroxidative stress. These data support the view that NO and ONOO$^-$ interact in vitro to modulate the adherence of cancer cells to endothelium. Thus, agents that enhance NO bioavailability or reduce nitroxidative stress may have therapeutic potential for improving endothelial function and reducing the risk of metastasis.
4.6. The imbalance of [NO]/[ONOO−] increases ICAM-1 and VCAM-1 expression on non-stimulated HLEC.

However, a fundamental question remains as to how NO and ONOO− regulate the adhesion of cancer cells. Several adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin, have been identified as being responsible for the endothelial adhesion of cancer cells (141). NO may decrease ICAM-1 and VCAM-1 expression by inhibition of NF-κB activation and increasing the expression and nuclear translocation of IkappaBalpha (66). The attachment of circulating tumor cells to the endothelium is facilitated by the up-regulation of endothelial adhesion molecules and is a key event that leads to metastasis (142).

The expression of ICAM-1 was significantly down-regulated by approximately 40% by treatment with VAS2870 over a wide range of concentrations (Figure 3.29A). While a 25% and 50% decrease in VCAM-1 expression was observed with 0.0125mM and 0.025mM VAS2870 respectively. A 10% decrease in ICAM-1 and VCAM-1 expression was observed with MnTBAP over a wide range of concentrations (Figure 3.29B). Thus the inhibitory effect of VAS2870 and MnTBAP on the adhesion of cancer cells to non-stimulated HLEC may be via downregulation of ICAM-1 and VCAM-1 expression.

However, addition of PEG-SOD and SNAP has no significant effect on ICAM-1 or VCAM-1 expression. Thus, the inhibition of PEG-SOD and SNAP on cancer cell adherence to HLEC may be mediated via other specific adhesion molecules on endothelial cell or tumor cell surface.
4.7. The imbalance of \([\text{NO}] / [\text{ONOO}^-]\) increases ICAM-1 and VCAM-1 expression on TNF-\(\alpha\) stimulated HLEC

This study demonstrated significant increases in the cell surface expression of VCAM-1 and ICAM-1 in response to 24 h treatment of HLECs with human recombinant TNF-\(\alpha\) (1ng/ml). However, the stimulatory effect of TNF-\(\alpha\) was abolished in the presence of NO and antioxidants, which attenuate TNF-\(\alpha\)-induced ICAM-1 and VCAM-1 expression to different extents. Exposure of HLECs to VAS2870, a NADPH oxidase inhibitor, most effectively inhibited endothelial surface expression of ICAM-1 and VCAM-1. Another two superoxide modulators PEG-SOD and MnTBAP also significantly inhibited TNF-\(\alpha\)-induced VCAM-1 expression. Additionally, the incubation with SIN-1, a ONOO\(^-\) donor, increased TNF-\(\alpha\)-stimulated ICAM-1 and VCAM-1 expression by approximately 10% (P<0.05), which contribute to adhesion between the tumor cell and HLECs and may enhance metastatic potential. We also found that the endogenous production of NO by L-arginine in HLEC only mildly inhibited TNF-\(\alpha\)-induced VCAM-1 expression by 5%, while it showed no effect on TNF-\(\alpha\)-induced ICAM-1 expression (Figure 3.33A). Thus, the inhibition of L-arginine on cancer cell adhesion to HLEC may not be mediated via ICAM-1 or VCAM-1 and may be affected by other specific proteins on endothelial cells. Similarly, the NO donor, SNAP, is not sufficient to inhibit the up-regulation of ICAM-1 and VCAM-1 in response to TNF-\(\alpha\) stimulation, indicating that higher concentrations of NO, in the pharmacological range, may be required to suppress the induction of ICAM-1 and VCAM-1.
A reasonable assumption of the current study is that the regulation of TNF-α–induced ICAM-1 and VCAM-1 expression is, at least in part, \([\text{NO}]/[\text{ONOO}^-]\) dependent. This is supported by our finding that superoxide modulators suppressed ICAM-1 and VCAM-1 expression while the \(\text{ONOO}^-\) donor increased ICAM-1 and VCAM-1 expression. One can assume that \([\text{NO}]/[\text{ONOO}^-]\) regulates CAM expression, or HLEC adhesiveness, by affecting endothelial cell oxidative/nitroxidative stress. Indeed, we found that increased \([\text{NO}]/[\text{ONOO}^-]\) level, or decreased oxidative stress, was associated with deceased cancer cell adhesion. This is consistent with another study showing that decreased intracellular oxidative stress by NO result in down-regulation of VCAM-1 expression through NF-κB-regulated transcriptional pathways (60). Our data also show that the levels of ICAM-1 and VCAM-1 expression positively correlated with metastatic potential of CACO-2 and HT1080 cells. Down-regulation of ICAM-1 and VCAM-1 at the protein levels strongly inhibited CACO-2 and HT1080 cell adhesion. Hence, we propose that down-regulation of ICAM-1 and VCAM-1 expression could be one mechanism by which NO blocks the capabilities of CACO-2 and HT1080 cells to adhere to HLECs and consequently preventing their metastatic potential.

4.8. Tumor cell lines differ in their adhesive preference for the endothelium

We also compared the adhesion patterns of three cancer cell lines (CACO-2, HT1080, MCF-7) to HUVECs and HLECs under static and flow conditions. Our data show that the three tumor cell lines differed in their adhesive preference for endothelial cells, indicating the heterogeneous nature of tumor cell adhesion.
Adhesion of CACO-2 cells was greater to HUVEC than HLEC under static conditions, while HT1080 cells exhibited a marked preferential adherence to HLEC. However, the adhesion of MCF-7 breast cancer cells to either HLECs or HUVECs is not likely to occur under static conditions. This is in accordance with the \([\text{NO}]/[\text{ONOO}^-]\) data showing that exposure of HLECs to HT1080 cells resulted in the lowest \([\text{NO}]/[\text{ONOO}^-]\) ratio (0.79±0.14, at 1×10^6 cells/ml) among the three cancer cell lines, indicative of dysfunctional endothelium and severe oxidative/nitrooxidative stress in HLEC induced by HT1080 cells. MCF-7 cells are larger than CACO-2 and HT1080 cells, as examined by microscopy. Compared with CACO-2 and HT1080 cells, it is more difficult to detach MCF-7 cells from tissue culture treated flasks after trypsin incubation. Trypsin is a proteolytic enzyme which is known to break down the adhesion proteins enabling the cells to adhere to the vessel (143). Therefore, MCF-7 cells may lack certain surface proteins which are required to interact with trypsin. These proteins may also be required to interact the endothelial cells. This may explained why the adhesion of MCF-7 cells to either HLECs or HUVECs is not likely to occur under static conditions. These results also indicate that cancer cell adhesion depends on the adhesive properties of both tumor cells and the endothelial cell substrate.

However, our data show that CACO-2 cells exhibited higher levels of adhesion to TNF-α-stimulated HLEC as compared to HT1080 cells under flow condition, which is opposite to the pattern observed under static condition. This may be explained by the fact that we used a higher concentration of TNF-α (50 vs.1ng/ml) under flow condition, indicating that CACO-2 cell adhesion may be greatly enhanced by higher levels of
inflammation. Alternatively, although HT1080 cells had a greater adherence to HLEC under static condition, the binding between HT1080 cells and the endothelium is not strong enough to withstand the shear stress of 0.5dynes/cm² under study, therefore the weakly binding cancer cells were washed away resulting in decreased adhesion. Again, the adhesion of MCF-7 breast cancer cell to lymphatic endothelium is not likely to occur under flow conditions, suggesting that MCF-7 may lack adhesion receptors on the cell surface which are required to interact with specific ligands on the lymphatic endothelium.

These results demonstrate that the ability of cancer cells to adhere to the lymphatic endothelium varies. Although the molecular mechanisms are not clear, this phenomenon may explain the preferential lymphatic spread of some tumors. Different binding efficiencies may have consequences in the metastatic potential of cancer cells in vivo.

4.9. TNF-α contributes to cancer cell adhesion under flow condition

Our findings support the hypothesis that interaction of circulating tumor cells with lymphatic endothelium may be enhanced at sites of inflammation. We found that only a few CACO-2 or HT1080 cells adhered to non-stimulated HLEC under flow conditions. However, increased adhesion was observed when HLECs were pre-incubated with TNF-α. There is a steady increase in the adhesion of cancer cells with increased concentration of TNF-α in the range of 1ng/ml to 50ng/ml. However, the number of adherent cells reached a plateau when the concentration of TNF-α is within the range of 50ng/ml to 500ng/ml. Our finding is consistent with previous studies showing that the
adhesion of cancer cells to lymphatic endothelium was augmented by inflammatory stimulus such as IL1-β or TNF-α (24). Similarly, TNF-α stimulation of both human dermal lymphatic endothelial cells and tumor cells significantly increased tumor-endothelial cell adhesion under static condition (25). It has been suggested that pro-inflammatory factors such as TNF-α, interleukin-1, and lipopolysaccharide may promote tumor cell adhesion to endothelial cells through up-regulation of adhesion molecules such as ICAM-1 and VCAM-1 on both cell types (65), probably via the activation of NF-κB (66).

4.10. Low wall shear stress facilitates cancer cell adhesion

Using a flow chamber, we examined cancer cell adhesion to TNF-α-stimulated HLECs under flow conditions. In this system, HLECs were cultured on petri dishes and subjected to laminar shear stress of 0.5 dynes/cm², analogous to that found in the lymphatic circulation. Our data showed that slight changes in shear stress can cause large changes in cancer cell adhesion to TNF-α-stimulated HLEC. Increasing the wall shear stress from 0.1 to 0.5 dynes/cm² reduced the number of adherent CACO-2 cells by a factor of two on TNF-α-treated endothelium. Similar results were observed with HT1080 cell adhesion.

Our findings support the hypothesis that low levels of shear stress may enhance the interaction of circulating tumor cells with the endothelium. Shear stress exerted by flowing blood and interstitial fluid modulate the behavior of circulating tumor cells (31). The generation of NO may decrease cancer cell adhesion by inducing vessel dilation and
increasing volumetric flow rate. Initial studies have shown that $\text{O}_2^-$ may act as a vasoconstrictor through mobilization of cytosolic $\text{Ca}^{2+}$ in vascular smooth muscle cells or promote $\text{Ca}^{2+}$ sensitization of the contractile elements (144). Another study also indicates that oxidative stress causes vasoconstriction of the vessels and increases in blood pressure by reducing the bioavailability of NO in the cardiovascular system (145). It is possible that NO supplementation could directly induce vasodilation by reducing $\text{O}_2^-$ concentration and oxidative stress, thereby enhancing wall shear rate and decrease the likelihood of cancer cell adherence. In addition, previous studies indicate that $\text{ONOO}^-$ can cause vasoconstriction and increase vascular resistances (87,88) through inhibition of vascular smooth muscle ATP-sensitive potassium channel activation (97). Therefore the imbalance of $\text{[NO]/[ONOO}^-\text{]}$ may regulate cancer cell adhesion through inhibition of vasodilatation and increasing vascular resistances. Furthermore, shear stress may also change local concentration gradients of signaling proteins such as chemokines and growth factors, which in turn may regulate cancer cell adhesion.
5. Conclusion

In summary, this study clearly demonstrated, for the first time, that the balance between [NO] and [ONOO⁻] is a main factor in modulation of the adhesion of human CACO-2 and HT1080 cancer cells to HLECs. The modulation mechanisms may include: inhibition of cancer cell adhesion, down-regulation of CAM expression and possibly vasodilation. We demonstrated that frequent collision of cancer cells with the endothelium caused an acute increase in oxidative stress and diminished NO level which facilitates cancer cell adhesion through activation of endothelial CAM expression. The restoration of endothelial function and a level of [NO]/[ONOO⁻] by using antioxidants and NOS cofactor decreased cancer cell adhesion to the endothelium through reduction of cellular sources of oxidative stress and inhibition of ICAM-1 and VCAM-1 expression. Among them, VAS2870 most effectively increased [NO]/[ONOO⁻] balance and down-regulated cancer cell adhesion on HLECs under static conditions. Hence, the use of agents that promote the formation of bioavailable NO and diminish the production of ONOO⁻ may provide new therapies to suppress metastasis by decreasing tumor cell adhesion to the endothelium. Our results also showed clearly that the likelihood for tumor cells to adhere to the lymphatic endothelium is greatly enhanced by inflammatory reactions and/or reduced blood flow. NO supplementation can directly induce vasodilation by reducing oxidative stress, thereby enhancing wall shear rate and decrease the likelihood of cancer cell adherence. Thus, our current study identifies bioavailable NO as a potential therapeutic candidate that reduces colon and fibrosarcoma metastasis by modulating vasodilatation and the expression of adhesion molecules. Also, this study
identifies ONOO$^-$ as a promoter of cancer cell adhesion. Therefore, the balance of [NO]/[ONOO$^-$] can be used to diagnose defense capability of endothelium against cancer cell metastasis. In addition, the restoration of endothelial function with drugs which enhance production of bioavailable NO and/or reduce cytotoxic ONOO$^-$ may be of significant importance in clinics to prevent cancer metastasis.

Figure 5.1 The collision of cancer cells induced oxidative stress in the endothelium.
Figure 5.2. Schematic illustration of the role of the [NO]/[ONOO•⁻] balance in lymphatic metastasis.
6. Future Work

Current study focused on the effect of NO and ONOO$^-$ on ICAM-1 and VCAM-1 expression on lymphatic endothelial cell surface. Because cancer cell-lymphatic endothelial cell interaction is modulated by the amount of adhesion molecules on both cell types, it will be interesting, in future work, to fully elucidate the effect of NO and ONOO$^-$ on CAM expression on cancer cell surface as well. In addition, adhesion molecules other than ICAM-1 and VCAM-1 may also be involved in the adhesion process. Thus, the expression of other adhesion molecules such as E-selectin should also be examined.

Furthermore, chemokines and lymphangiogenesis factors may also play an important role in lymphatic metastasis (7). It will be interesting to study the effect of NO and ONOO$^-$ on chemokines and lymphangiogenesis factors in cancer cell-lymphatic endothelial cell interaction.

Besides, different tumor cells lines should be examined to precisely delineate the role of NO/ONOO$^-$ in lymphatic metastasis of specific forms of tumors. In addition, results from in vitro experiments can be used to predict the situation in vivo, but they can’t replace animal experiments completely. In vivo experiments are needed in order to find out the roles of NO and ONOO$^-$ in tumor cell adhesion.
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