Forum on Immunopathological Diseases and Therapeutics

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AIMS AND SCOPE

The aim of this journal is to serve as the publication vehicle for a workshop series which focuses on an important and timely gene product or agent that is critical in the pathogenesis, progression and disease manifestation. Additionally, the gene product could serve as a potential biomarker for prognosis and diagnosis, and also as an object for therapeutic intervention and for development of novel specific and targeted therapeutics.

The workshops cover topics that include biochemistry, immunology, molecular biology, genetics, molecular and cellular mechanisms of disease, clinical studies and new innovative therapies. A given workshop is highly focused and invited speakers are selected from active investigators who are leaders in the field of the topic of each workshop. The workshop consists of individualized sessions and each session is concluded with a discussion and expert opinions and recommendations made by other invited experts in light of current progress in the filed.

This journal publishes the main presentations and expert opinions of each workshop in a special issue in the series. The authors of the articles (and workshop participants) are chosen from a small number of basic scientists and clinical investigators. As a result, each issue of the journal will deal with a particular subject. The manuscripts are reviewed in the usual manner by guest editors and by the series Editors. The special issue will be published no later than three months after final review by the series Editors. A major goal of the journal is to attain further integration of the various presentations and discussion topics.

SPECIAL ISSUE

FIRST INTERNATIONAL WORKSHOP ON "NITRIC OXIDE IN CANCER THERAPY"

HELD IN DIJON, FRANCE SEPTEMBER 11-12, 2009

THE FIRST INTERNATIONAL WORKSHOP ON "NITRIC OXIDE AND CANCER THERAPY" WAS FOCUSED ON THE CURRENT STATUS OF NITRIC OXIDE (NO) APPLICATIONS IN CANCER THERAPY. ALTHOUGH THE APPLICATION OF NO HAS BEEN REPORTED IN VARIOUS MANIFESTATIONS INCLUDING IMMUNE RESPONSES, VASCULAR DISEASES AND PREVENTION, HOWEVER, THE ROLE OF NO IN CANCER THERAPY HAS ONLY RECENTLY BEEN RECOGNIZED. THIS WORKSHOP, THEREFORE, EMPHASIZED THE POTENTIAL APPLICATION OF NO AND ALSO VARIOUS APPROACHES USED TO DELIVER AND/OR INDUCE NO IN CANCER CELLS. NOTEWORTHY, CLINICAL STUDIES IN HUMANS HAVE BE PRESENTED AND DEMONSTRATED POTENTIAL THERAPEUTIC EFFICACY OF NO IN CANCER PATIENTS. THE PROCEEDINGS OF THIS WORKSHOP ARE PUBLISHED IN TWO PARTS, VOLUME I, ISSUE 3 (PART 1) AND ISSUE 4 (PART 2).

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JEAN-FRANÇOIS JEANNIN

Director EPHE Tumor Immunology and Immunotherapy Laboratory
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First International Workshop on
“Nitric Oxide in Cancer Therapy”

Guest Editor

Professor Jean-François Jeannin

Jean-François Jeannin is Professor of Immunology at Ecole Pratique des Hautes Etudes (EPHE) and director of the EPHE Tumor Immunology and Immunotherapy Laboratory, an INSERM (National Institute of Health and Medical Research) team. His main research interests have included the effects of lipopolysaccharides in the tumor immune response and the immunotherapy of cancer with lipid A. Currently, he is investigating mechanisms of immunotherapy with synthetic lipid A analogs in cancer patients and animal cancer models. He is especially interested in the sensitization of tumor cell death by nitric oxide produced in tumors during lipid A immunotherapy. Jean-François Jeannin is Dean of the Life Sciences Department of EPHE.

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Cross-Regulation Between Wnt and NF-κB Signaling Pathways

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ABSTRACT: Cross-regulation between the Wnt and nuclear factor (NF)-κB signaling pathways has emerged as an important area for the regulation of a diverse array of genes and pathways active in chronic inflammation, immunity, development, and tumorigenesis. The ligands, kinases, transcription factors, and products of their target gene expression are involved in cross-regulation of these two signaling pathways. Both β-catenin and NF-κB activate inducible nitric oxide synthase (iNOS) gene expression; however, β-catenin also exerts an inhibitory effect on NF-κB-mediated transcriptional activation, including iNOS. The recent discovery of functional cross-regulation between these two pathways has shown complex roles for Wnt/β-catenin and NF-κB signaling in the pathogenesis of certain cancers and other diseases. This review focuses on the molecular mechanisms of cross-regulation between Wnt/β-catenin and NF-κB signaling pathways in cancer cells.

KEY WORDS: Wnt signaling pathway, NF-κB signaling pathway, cross-regulation, β-catenin

I. INTRODUCTION

Cross-regulation of several cellular signaling pathways has been shown to play important roles in modifying the biological effects of gene expression. Wnt/β-catenin and nuclear factor (NF)-κB are independent pathways involving the regulation of many physiological and pathological effects related to the areas of development, immune function, inflammation, tumorigenesis, tumor invasion, and metastasis, as well as cardiovascular and bone diseases. However, the activity and signaling consequences are also regulated by direct interactions between these two pathways, which results in diversity and complexity.

Many reports demonstrate that these two pathways independently initiate oncogenesis in colon, liver, and other organs. A few recent studies have shed light on the cross-regulation between these two pathways

that influences development and carcinogenesis. Because these two pathways are involved in the regulation of gene expression and activation, transcription factors are highly active in most cancer cells, and thus are ideally suited for development of anticancer drug therapies. Several transcription factors, including β-catenin/Tcf and NF-κB, are promising targets for cancer therapeutics.[1] It is now evident that strategies targeting the cross-regulation between these pathways may be a promising direction for future cancer therapeutics.

The molecular basis of the cross-regulation between the Wnt and NF-κB signaling pathways is an essential issue for fully understanding potential therapeutic mechanisms. In order to clearly describe the interplay between Wnt and NF-κB pathways, the mode for cross-regulation between the two was recently reviewed by Guo and Wang,[2] who applied three basic modes of signaling cross-regulation to elucidate the commonalities. A cross-regulation exists between designated pathways A and B when both of the following criteria are met: i) functionally, the combinatorial signal from A and B must produce a different response than that triggered by A or B alone; and ii) mechanistically, A and B pathways must be connected in at least one of three ways: (a) components of the two pathways physically interact, (b) components of one pathway are enzymatic or transcriptional targets of the other, and (c) one signal modulates or competes for a key modulator or mediator of the other. Moreover, we will also consider the dynamic manner in which canonical or non-canonical Wnt signaling is reciprocally regulated with the NF-κB signaling pathway. Although many commonalities exist by which Wnt signaling interplays with the NF-κB pathway, the analyses of cell line- and tissue-specific differences are still wanting.[2]

In this review, we summarize the mechanisms of cross-regulation between Wnt and NF-κB signal transduction pathways focusing on cross-modulation and reciprocal modulation. For the functional cross-regulation between Wnt and NF-κB signaling pathways, in addition to the marquis proteins β-catenin and NF-κB, we will also address the roles of E-cadherin, Wnt proteins, Wnt antagonists, glycogen synthase (GSK)-3β, inhibitor of NF-κB (IκB)/IκB kinase (IKK), and β-transducin repeat-containing protein (β-TrCP). These components are involved in the regulation of transcriptional activity or degradation cascades of these two pathways.

II. WNT SIGNALING PATHWAY

Currently, Wnt signaling is composed of Wnt/β-catenin (also referred to as canonical Wnt) and non-canonical Wnt signaling is based on the

absence of β-catenin involvement. In response to Wnt protein binding to its receptor complex, signal transduction is triggered under physiologic conditions. Constitutively activated β-catenin signaling due to β-catenin mutation or adenomatous polyposis coli (APC) gene deficiency results in evasion of the degradation complex, and has been observed and in colon cancer and other tumors.

II.A. Wnt Proteins

The Wnt proteins are secreted, lipid-modified signaling molecules that control a number of central cellular processes. There are 19 Wnt proteins that have been identified in mammals so far (Wnt homepage: http://www.stanford.edu/~rnusse/wntwindow.html). Some, such as Wnt1 and Wnt3, either activate or inhibit the canonical Wnt signaling pathway, and some, including Wnt5A, Wnt5B, and Wnt11, activate the non-canonical Wnt signaling pathway.[3] The combinations of Wnt proteins (ligands) and their receptors in Wnt signaling have been summarized previously by Kikuchi et al.[4]

II.B. Wnt/β-Catenin Signaling

In a cell, β-catenin is localized to the transmembrane-, cytoplasmic-, or nuclear-β-catenin pools. The first two pools determine the physiological role in development and homeostasis associating with the cell-cell adherence protein E-cadherin, while the nuclear-β-catenin fraction is involved in oncogenesis associated with the tumor suppressor gene product APC. Wnt/β-catenin signaling is mediated by β-catenin, which plays a dual role as a transcription factor and as a molecule of cell adherence junctions interacting with the cadherins. Wnt/β-catenin signaling has diverse functions in regulating cellular processes such as proliferation, differentiation, migration, and survival, whereas non-canonical Wnt signaling controls tissue polarity and movement. In the absence of Wnt, cytosolic β-catenin protein is constantly degraded by the β-catenin destruction complex, which is composed of Axin, APC, GSK-3β, and casein kinase 1 (CKI). In the absence of Wnt, CKI and GSK-3β sequentially phosphorylate the amino-terminal region of β-catenin, leading to recognition by β-TrCP, an E3 ubiquitin ligase subunit resulting in poly-ubiquitination and degradation. This continual turnover of β-catenin silences the Wnt pathway. When the Wnt ligand binds to its receptor, Frizzled (Fz), and its co-receptor, low-density lipoprotein receptor-related protein 5/6 (LRP5/6), Wnt, Fz, and LRP6 form a complex, together with an

intracellular protein Dishevelled (Dvl), and in turn phosphorylate LRP6. These molecular events prevent β-catenin phosphorylation and degradation. The stabilized β-catenin is accumulated in the cytoplasm and travels to the nucleus, where β-catenin binds the Tcf/Lef (T-cell transcription factor/lymphocyte enhancer factor) family of transcription factors and activates the Wnt target gene expression. In a cancer cell, a component of Wnt signaling such as APC or β-catenin is mutated. In this case, β-catenin can be stabilized in the cytoplasm and works as a co-activator of Wnt/β-catenin signaling involved in many aspects of tumorigenesis, cancer development, and progression.[5-7]

II.C. Non-canonical Wnt Signaling

When Wnt proteins bind to their receptor, there are two branches of the non-canonical Wnt signaling pathway. The first is generally called as the Wnt/c-Jun N-terminal kinase (JNK) pathway, which activates small GTPases such as Rac, Rho, and CDC42 and, more downstream, Rho-kinase (ROCK) or JNK. The other Wnt-mediated non-canonical signaling pathway stimulates the intracellular increase in Ca^{2+}, possibly mediated by G-proteins. This pathway activates several downstream targets, including protein kinase C (PKC), and Ca-calmodulin kinase II (CaMKII). The elevated levels of Ca^{2+} can activate the phosphatase calcineurin, which induces the dephosphorylation of the transcription factor nuclear factor of activated T-cells (NFAT), resulting in an accumulation of NFAT in the nucleus and an activation of target genes. The effects of non-canonical Wnt signaling are in tissue polarity control and cell migration.[8,9]

II.D. Wnt Antagonists

Several secreted protein families inhibit or mediate Wnt signaling: i) secreted Fz-related proteins (sREPs) and Wnt inhibitory protein (WIF) bind Wnt or Fz as inhibitors of canonical and non-canonical Wnt signaling; ii) the Dickkopf (DKK) and the WISE/SOST families are LRP5/6 ligands/antagonists: DKK1 inhibits Wnt signaling via inducing LRP6 internalization/degradation through transmembrane kremen protein, and SOST is able to disrupt Wnt-induced Fz-LRP6 complex in vitro; iii) Shisa proteins trap Fz proteins in the endoplasmic reticulum and prevent Fz from reaching the cell surface; and iv) *Xenopus cerberus* and Nodal and bone morphogenetic protein (BMP) binds to and inhibits Wnt signaling.[7]

III. NF–κB SIGNALING PATHWAY

The NF-κB transcription factors are generally retained in the cytoplasm of resting cells, and when activated bind to a large array of enhancer sequences (over 150 genes) that are present in most (if not all) cells. Mammalian NF-κB transcription factors consist of five homologous subunits (RelA/p65, c-Rel, RelB, p50/ NF-κB1, and p52/ NF-κB2) that dimerize and are held in the cytoplasm by specific proteins, the IκBs. Immediately upstream from the IκB-bound NF-κB dimers is the IKK complex, comprised of two catalytic (IKKα and IKKβ) and one regulatory (IKKγ/ NF-κB essential modulator [NEMO]) subunits. Several pathways of cell stimulation converge to activate the IKK complex, which then phosphorylates NF-κB-bound IκB proteins that targets the IκB protein for ubiquitination and degradation by the 26S proteasome by creating a binding site for Skp1-Cullin1-F-box protein (SCF)/β-TrCP ubiquitin ligase complex. The liberated NF-κB translocates into the nucleus and engages transcriptional programs. For activation of NF-κB signaling, the two most recognized pathways are the so-called "classical" and "alternative" pathways. The former depends on NEMO, IKKβ activation, and nuclear localization of RelA/p50 dimers, and is associated with inflammation, while the latter depends on IKKα activation, probably via the upstream NF-κB-inducing kinase (NIK) and nuclear localization of p52/RelB heterodimers, and is important in lymphoid organogenesis. Both pathways of NF-κB activation have now been implicated in carcinogenesis.[10,11]

IV. CROSS-REGULATION OF WNT AND NF-κB PATHWAYS

IV.A. Wnts and Cross-regulation Between Wnt and NF-κB Pathways

In recent years, research studies have shown important roles for Wnt5A. The functions of Wnt5 signaling are in bridging innate and adaptive immunity to infections, and Wnt5A is a cancer-related gene involving in invasion and metastasis of many cancers.[12] Wnt5A transcription is regulated by many proteins, included NF-κB. A conserved NF-κB-binding site within the Wnt5A promoter B region elucidates the mechanisms by which tumor necrosis factor-alpha (TNFα) and Toll-like receptor (TLR) signals up-regulate Wnt5A via MAP3K7 signals. SNAI1 (Snail), CD44, G3BP2, and YAP1 are Wnt5A signaling target genes.[13,14] Following stimulation of macrophages with different mycobacterial species and conserved bacterial structures, Wnt5A is

expressed, which involves the activation of TLR signaling and NF-κB. Induction of Fz5, the Wnt5A receptor, has also been reported in human peripheral-blood mononuclear cells. Binding to its receptor, Wnt5A activates canonical and non-canonical Wnt signaling pathways and plays key roles in a variety of cellular processes during development and carcinogenesis. Importantly, the expression of Wnt5A protein is controlled by the NF-κB signaling pathway, which may be implicated as an essential mediator not only for infection, but also for cancer development.

Binding with its receptor, Wnt-11 signaling is sufficient to inhibit not only the canonical Wnt but also JNK/activator protein-1 (AP-1) and NF-κB signaling in Chinese hamster ovary (CHO) cells, thus serving as a non-canonical Wnt ligand in this system and leading to the promotion of cell viability.[3]

WntD is a member of the *Drosophila* Wnt family. Toll/NF-κB signaling has an evolutionarily conserved role in regulating innate immunity. WntD acts as a feedback inhibitor of the NF-κB homolog *Dorsal* during both embryonic patterning and in the innate immune response to infection. WntD expression is under the control of *Toll/Dorsal* signaling, and increased levels of WntD block *Dorsal* nuclear accumulation, even in the absence of the IκB homolog Cactus. Thus, the WntD signal is independent of the common Wnt signaling component Armadillo (β-catenin), and WntD serves as a feedback antagonist of *Toll* signaling and maintaining low basal levels of *Toll/Dorsal* signaling in the fly. Moreover, WntD mutants show defects in embryonic *Dorsal* regulation and in the adult innate immune system.[15]

IV.B. Wnt Antagonists Affect the Cross-regulation Between Wnt and NF-κB Pathways

DKK1 is a secreted Wnt antagonist whose transcription is mediated by canonical Wnt signaling.[16-18] The activation of Wnt signaling and overexpression of DKK1 have been observed in breast cancer. It is interesting that human breast cancer cell lines that preferentially form osteolytic bone metastasis exhibited increased levels of Wnt signaling and DKK1 expression. Breast cancer cell-produced DKK1 blocks Wnt3A-induced osteroblastic differentiation and osteroprotegerin (OPG) expression, and Wnt3A-induced NF-κB ligand reduction. These results suggest that breast cancer-produced DKK1 may be an important mechanistic link between primary breast tumors and secondary osteolytic bone metastases.[19] In postnatal and adult life, osteoblasts and osteoclasts play opposite roles for bone matrix

formation and resorption. The interaction of these two cell types determines bone density. Numerous lines of evidence from genetic studies show that Wnt/β-catenin signaling regulates bone mass and bone diseases.[5] However, Wnt/β-catenin signaling promotes the activity of osteoblasts. It is clear that the decreased activity of osteoblasts contributes to osteolytic lesions in multiple myeloma. The production of DKK1 by multiple melanoma cells inhibits osteoblast activity. However, a neutralizing antibody (BHQ880) to DKK1 up-regulates the β-catenin level while down-regulating NF-κB activity in bone marrow stromal cells (BMSCs), and inhibits multiple myeloma cell growth in the severe combined immunodeficiency (SCID)-hu murine model. These results confirm DKK1 as an important therapeutic target in myeloma, and provide the rationale for clinical evaluation of BHQ880 to improve bone disease and to inhibit multiple myeloma growth.[20]

IV.C. E-Cadherin Mediates the Cross-regulation Between Wnt and NF-κB Pathways

E-cadherin plays a dual role in cells: in addition to its structural role in adherens junctions, E-cadherin mediates the dynamic of β-catenin, which acts as a transcription factor in the nucleus by serving as a coactivator of the Tcf/Lef family of DNA-binding proteins. On the other hand, E-cadherin, a target gene of Wnt/β-catenin signaling,[21] is involved in the negative regulation of canonical Wnt signaling. Because the molecular basis for the interaction of β-catenin with cadherins and Tcf/Lef family members is mediated by the same domain on the β-catenin molecule (the so-called arm repeat), these interactions are mutually exclusive. Thus, recruitment of β-catenin into adherens junctions by elevating the expression of cadherin can decrease its nuclear pool and antagonize β-catenin–Tcf/Lef transactivation.[22]

Functional cross-regulation between Wnt and NF-κB pathways also occurs during epithelial-mesenchymal transition (EMT) mediated by E-cadherin and its transcriptional repressor Snail. Expression of Snail promotes the conversion of epithelial cells to mesenchymal cells, and occurs concomitantly with the down-regulation of E-cadherin and the up-regulation of expression of mesenchymal genes, such as those encoding fibronectin and Lef1.[23] E-cadherin overexpression decreased the transcriptional activity of the fibronectin promoter and reduced the interaction of β-catenin and NF-κB with this promoter. Fibronectin is a target gene of Wnt signaling.[24] Fibronectin and Lef1 gene expressions are dependent on the transcriptional activity of β-catenin and NF-κB. These activities are both controlled by the pres-

ence of E-cadherin-dependent cell contacts in epithelial cells. Similar to β-catenin, NF-κB is found to be physically associated with E-cadherin and other cell-adhesion components. Interaction of the NF-κB p65 subunit with E-cadherin or β-catenin is reduced when adherens junctions are disrupted by K-ras overexpression or by E-cadherin depletion using small interfering RNA (siRNA). E-cadherin, as a Wnt target gene, not only controls the transcriptional activity of β-catenin, but also that of NF-κB during EMT. Binding of NF-κB to the adherens-junctional complex prevents the transcription of mesenchymal genes.[25] The major route for signal transduction by E-cadherin involves the negative-feedback regulation of β-catenin-Tcf signaling and down-regulation of NF-κB. It is expected that NF-κB transcriptional activity is mainly inhibited by the adherens-junction-associated pool of β-catenin.[25] Furthermore, malignant transformation of melanocytes frequently coincides with the loss of E-cadherin expression. Melanoma cells show constitutively active NF-κB, whereas no such activity is found in primary melanocytes. The mechanism for loss of E-cadherin leading to induction of NF-κB activity in melanoma cell lines has been proposed to be due to cytoplasmic β-catenin inducing p38-mediated NF-κB activation in malignant melanoma.[26]

IV.D. GSK-3β Mediates the Cross-regulation Between Wnt and NF–κB Pathways

GSK-3β has emerged as one of the most attractive therapeutic targets for the treatment of many diseases and disorders. GSK-3β plays dual roles in the APC-β-catenin destruction complex in regulating Wnt signaling and as a critical regulator of NF-κB activity, including gene transcription, cell cycle, apoptosis, inflammation, glucose metabolism, stem-cell renewal, and differentiation.[27] Targeting GSK-3β is a promising approach for cancer therapy.[28] Deregulated GSK-3β activity in colorectal cancer is associated with tumor cell survival and proliferation. The inhibition of GSK-3β has been observed in many tumors,[29] and activates canonical Wnt and NF-κB signaling pathways. Specifically, GSK-3β controls the degradation of β-catenin by phosphorylating β-catenin at Ser^{37} and Ser^{33}. These phosphorylations provide a binding site for the E3 ubiquitin ligase β-TrCP, leading β-catenin to the proteasome complex for degradation. In this respect, GSK-3β is a negative regulator of Wnt signaling. On the other hand, GSK-3β positively regulates NF-κB by mediating the degradation of IκB, a central inhibitor of NF-κB.[30,31] Inhibition of GSK-3β differentially modulates NF-κB, cAMP response element-binding protein (CREB), AP-1, and

β-catenin signaling in mouse primary hepatocytes, but fails to promote TNFα-induced apoptosis. Stimulation of canonical Wnt signaling and CREB activity led to up-regulated levels of anti-apoptotic factor.[32] These observations indicate a complex cross-regulation between NF-κB and β-catenin pathways.

In collaboration with Perwez Hussain, we have shown that the human inducible nitric oxide synthase (iNOS or NOSII) gene is a target of the Wnt signaling pathway.[33] Two functional Tcf-4 binding elements (TBE1 and TBE2) were identified upstream in the human iNOS promoter. Overexpression of β-catenin and Tcf4 significantly increased both basal and cytokine-induced human iNOS promoter activity, and the induction was dependent on intact TBE sites. Furthermore, overexpression of β-catenin or TCF4 increased iNOS mRNA and protein expression in HCT-116 cells. Lithium chloride, an inhibitor of GSK-3β, increased the cytosolic β-catenin level, iNOS expression, and nitric oxide (NO) production in primary human and rat hepatocytes and cancer cell lines. In vivo, lithium chloride also increased hepatic β-catenin level in a dose-dependent manner with simultaneous increase in iNOS expression. These findings support the hypothesis that β-catenin up-regulates iNOS, and suggests a novel mechanism by which the Wnt/β-catenin signaling pathway contributes to cancer by increasing NO production (Fig. 1).

Other research has shown that E-cadherin disassembly and concomitant inactivation of GSK-3β that induces β-catenin triggered NF-κB-dependent up-regulation of iNOS in hepatocytes.[34] GSK-3β/APC may also regulate NF-κB activity with an inverse correlation in vitro and in vivo through β-catenin by cross-regulating with NF-κB signaling pathway.[35]

A study of the associations between these two pathways during trans-differentiation was conducted. The β-catenin/Tcf4/p300 signaling loops play an important role in trans-differentiation toward the morular phenotype of endometrial carcinomas. Cross-regulation between NF-κB/p65 and β-catenin/Tcf4/p300 signaling pathways through alterations in GSK-3β expression during trans-differentiation of endometrial carcinoma cells was noted. These findings provide evidence that a shift from NF-κB to β-catenin signaling pathways through alterations in GSK-3β expression may be essential for the induction of trans-differentiation of endometrial carcinoma cells.[36]

Signaling pathway cross-regulation does not just occur inside cells, but is also an intercellular event. Macrophage cells have a critical role in intestinal tumorigenesis. Because the activated macrophages caused by *Helicobacter* infection produce NF-κB-dependent

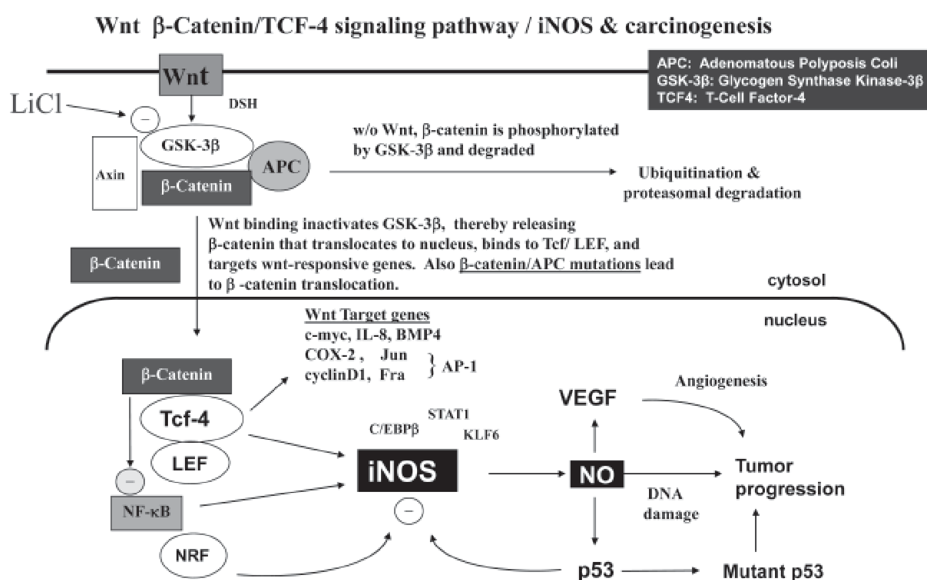

FIGURE 1. Summary of Wnt/β-catenin, NF-κB, and iNOS pathways. Both β-catenin and NF-κB activate iNOS gene expression, however, β-catenin also exerts an inhibitory effect on NF-κB-mediated transcriptional activation, including iNOS.

TNFα, which phosphorylates GSK-3β. The inactivated GSK-3β results in the stabilization of β-catenin, and promotes Wnt/β-catenin signaling in gastric cancer cells. This model is consistent with the observation that β-catenin nuclear accumulation in macrophage-infiltrated dysplastic mucosa of the K19-Wnt1 mouse stomach.[37] This study provides strong evidence that TNFα is a link between the chronic inflammation and promotion of preexisting Wnt/β-catenin signaling during tumorigenesis of gastric cancers.[37,38]

IV.E. IKKs/IκB and the Cross-regulation Between Wnt and NF-κB Pathways

Two kinases, IKKα and IKKβ, are critical activators of the NF-κB pathway. They are also important in the regulation of β-catenin function. IKKβ decreases β-catenin-dependent transcriptional activation, while IKKα increases β-catenin-dependent transcriptional activity in IKKα- and IKKβ-deficient mouse embryo fibroblasts[39] and in human multiple myeloma.[40] IKKα and IKKβ interacting with and phosphorylating β-catenin may in part be responsible for regulating β-catenin protein levels and cellular localization and integrating signaling

events between the NF-κB and Wnt pathways.[39] Even if some multiple melanoma cell lines have constitutive classical NF-κB activity, and a subset of multiple melanoma cell lines shows alternative NF-κB activity, only IKKα down-regulation decreases the expression of β-catenin and aurora-A, which are known to mediate multiple melanoma cell growth and survival.[40] IKKα plays a pivotal role in the regulation of β-catenin signaling through different mechanisms. First, IKKα can inhibit β-catenin degradation mediated not only by the Axin/APC/GSK-3β complex, but also by the Siah-1 pathway. Consistently, IKKα abolished the inhibition of β-catenin/Tcf-dependent transcription by Siah-1. Furthermore, IKKα interacted with β-catenin and mediated β-catenin stabilization by inhibiting β-catenin ubiquitination, which in turn stimulated β-catenin/Tcf-dependent transcription[41] Second, IKKα but not IKKβ, induces CyclinD1 expression, which is identified as a target of Wnt/β-catenin signaling pathway, also through Tcf activity. The CyclinD1 gene functions as a point of convergence between the Wnt/β-catenin and IκB pathways in mitogenic signaling. Mitogenic induction of G(1)-S phase progression and CyclinD1 expression is PI3K dependent, and CyclinD1$(^{-/-})$ cells show reduced PI3K-dependent S-phase entry. PI3K-dependent induction of CyclinD1 is blocked by inhibitors of PI3K/Akt/IκB/IKKα or β-catenin signaling. A single Tcf site in the CyclinD1 promoter is required for induction by PI3K or IKKα.[42]

RelA (p65) also is involved in the down-regulation of the Wnt/β-catenin pathway. This suppression does not depend on the transacting transcriptional ability of RelA. Furthermore, RelA affects neither the nuclear import of β-catenin nor the DNA-binding ability of the β-catenin/Tcf complex, suggesting that NF-κB modifies this signaling pathway after the binding of the β-catenin/Tcf complex with target DNA.[43]

Previous studies have shown that NF-κB activation plays certain roles in mediating proliferation and anti-apoptosis in response to progastrin on pancreatic cancer cells[44] and on proximal colonic crypts of FAbp-PG mice.[45] β-catenin expression can be activated in colonic crypts of mice in response to chronic (Fabp-PG mice) and acute (wild-type FVB/N mice) progastrin stimulation.[46] Significant increases are observed in the relative levels of cellular and nuclear β-catenin and pbeta-cat45 in proximal colonic crypts of Fabp-PG mice compared with that in wild-type littermates. IKKα/β/NF-κB activates β-catenin signaling, because treatment of Fabp-PG mice with the NEMO peptide (an inhibitor of IKKα/β/NF-κB activation) significantly blocks increases in cellular/nuclear levels of total β-catenin, pβ-catenin Ser^{45},

and pβ-catenin Ser[552] in proximal colons. Cellular levels of pβ-catenin Ser[33,37] and Thr[41], however, increase in the proximal colon in response to NEMO, probably due to a significant increase in pGSK-3β Tyr[216], facilitating degradation of β-catenin. Distal colonic crypts were less responsive.[46,47] This suggests a functional cross-regulation between the NF-κB and β-catenin pathways, and that the activation of β-catenin may contribute to the hyperproliferative effects of progastrin on proximal colonic crypts.[46]

IV.F. Physical Interaction of β-Catenin and NF-κB Components and the Cross-regulation of Wnt and NF-κB Pathways

1. β-Catenin Physically Interacts with NF-κB Components and Inhibits NF-κB Target Gene Function

Gene transcription activity can be activated or inhibited by signal-induced β-catenin and NF-κB interaction between transcription factors on regulatory elements positioned near their target genes. β-catenin as a coactivator of canonical Wnt signaling has been intensively studied, including cross-regulation with the NF-κB pathway. A direct interaction between Wnt and NF-κB signaling pathways was reported in a pioneering study finding that β-catenin can physically complex with NF-κB, resulting in a reduction of NF-κB DNA binding, transactivation activity, and target gene expression in some cancer cells.[48] It is interesting that repressed NF-κB activity was observed in human colon cancer cells in which β-catenin is activated. Importantly, activated β-catenin was found to inhibit the expression of NF-κB target genes, including *Fas* and *Traf1*. Furthermore, a strong inverse correlation was identified between the expression levels of β-catenin and Fas in colon and breast tumor tissues, suggesting that β-catenin regulates NF-κB and its targets in vivo. These findings led to the suggestion that β-catenin may play an important role in oncogenesis through the cross-regulation of NF-κB.[48]

Another example of the cross-regulation of these two pathways is the fine-tuned regulation of human iNOS gene expression. Our studies demonstrate that the hiNOS gene is regulated and targeted by NF-κB[49] and the Wnt/β-catenin signaling pathway.[33] Recently, we also reported that Wnt/β-catenin signaling regulates cytokine- or TNFα-induced hiNOS expression through interaction with the NF-κB pathway. Our in vitro (colon and liver cancer cell models) and in vivo (hepatocellular carcinoma tissues) data show that β-catenin signaling inversely correlates with cytokine-induced hiNOS and other NF-κB-

dependent gene expression (*Fas* and *Traf1*). These findings underscore the complex role of Wnt/β-catenin, NF-κB, and iNOS signaling in the pathophysiology of inflammation-associated carcinogenesis (Fig. 1).[50] However, a different study demonstrated that not all NF-κB target genes are repressed by the increased expression of β-catenin.[51] This reveals distinct and gene-selective molecular strategies for the down-regulation of NF-κB target genes by β-catenin.

2. β-Catenin Physically Interacts with NF-κB Components and Synergizes NF-κB Target Gene Function

In an analysis of the expression of TNFα-induced C-reactive protein (CRP), the p50 subunit of NF-κB as a positive regulator was found to be responsible for the transcriptional activation of CRP, and β-catenin to enhance the expression of a CRP mRNA in concert with p50. This protein-protein interaction is required for CRP expression. Therefore, CRP is mediated by the cross-regulation between Wnt/β-catenin and NF-κB signaling pathways via β-catenin binding with the NF-κB p50 subunit.[52] It is interesting that this interaction can be disrupted by the β-catenin-binding RNA aptamer as a tool for studying protein-protein interaction within the transcription complex and for modulating the expression of a target gene. The RNA aptamer binds Armadillo repeats of β-catenin, is effective in disrupting protein-protein interaction between β-catenin and NF-κB (p50), and effectively reduces TNFα-induced transcription from the promoter of CRP regulated by β-catenin and NF-κB p50.[53]

3. β-Catenin/Reptin Complex Physically Interacts with NF-κB Components and Controls NF–κB Target Gene Function

In a study of the transcriptional regulation of the metastasis suppressor gene *KAI1* (Kangai 1), which is a target gene of NF-κB signaling,[54] *KAI1* expression was found to be regulated by the interaction between β-catenin or Tip60 with the NF-κB pathway at the *KAI1* promoter. β-catenin expression functionally inhibits KAI1 expression by β-catenin complexed with reptin, which displaces the Tip60 coactivator complex. Down-regulation of KAI1 in prostate cancer cells involves the inhibitory actions of β-catenin, along with a reptin chromatin remodeling complex. This inhibitory function of β-catenin-reptin requires both increased β-catenin expression and recruitment of histone deacetylase activity. The coordinated actions of β-catenin-reptin components that mediate the repressive state serve to antagonize a Tip60 coactivator

complex that is required for activation; the balance of these opposing complexes controls the expression of KAI1 and metastatic potential. The molecular mechanisms underlying the antagonistic regulation of β-catenin-reptin involve the binding of NF-κB p50/p50 with β-catenin and the reptin complex at the *KAI1* promoter in metastatic cells. This is a typical β-catenin interaction with NF-κB and down-regulates its target gene, *KAI1* expression occurring on the *KAI1* promoter.[51]

IV.G. Cross-regulation of Wnt and NF-κB Pathways Mediated by Transcriptional Complex

1. NLK Mediates the Cross-regulation Between Wnt/β-Catenin and NF-κB Pathways

A very important kinase, NEMO-like kinase (NLK), is involved in the cross-regulation of Wnt and NF-κB pathways. NLK can be activated by Wnt1 and non-canonical Wnt signaling,[55] and is a serine/threonine kinase. NLK suppresses not only the transcription activity of the β-catenin/Tcf complex through phosphorylation of Tcf,[56] which establishes a negative feedback mechanism for the regulation of Wnt signaling, but also the transcription co-activators of NF-κB, such as CREB binding protein (CBP)/p300, rather than NF-κB itself.[57] These results suggest that NLK is a key player in the cross-regulation between Wnt and NF-κB signaling pathways, and may suppress a wide range of gene expression, possibly through NLK phosphorylating the C-terminal domain of CBP.[57]

2. NF-κB Signaling Affects β–catenin Transcriptional Activity

Lef1 is coactivator of the β-catenin transcription factor complex. A conserved NF-κB-binding site between mouse and human was selected through a bioinformatics analysis and mapped to 14 kb upstream of *Lef1* transcription initiation site. Overexpression of Lef1 in cartilage tissue of osteoarthritic patients has been observed, along with NF-κB-mediated *Lef1* gene regulation in chondrocytes. Treatment of IL-1β augments *Lef1* up-regulation and nuclear translocation of NF-κB in chondrocytes. Lef1 expression was synergistically up-regulated by interactions of NF-κB with Lef1/β-catenin in the same cells. This implicates a pivotal role for NF-κB in Lef1 expression in arthritic chondrocytes and in cartilage degeneration.[58]

In a study of non-steroidal anti-inflammatory drugs (NSAIDs) repressing CRT (β-catenin/Tcf4-regulated transcription) in colorectal

cancer, the NSAID diclofenac and a methanol extract of *Polysiphonia japonica* inhibited Wnt/β-catenin signaling without altering the level of β-catenin protein, and reduced the expression of β-catenin/Tcf-dependent genes. Diclofenac and the *P. japonica* extract, on the other hand, induced degradation of IκBα, which increased free NF-κB in cells. Also, the ectopic expression of p65, which is a component of NF-κB, suppressed CRT. These findings suggest that diclofenac inhibits Wnt/β-catenin signaling via activation of NF-κB in colon cancer cells.[52,59]

IV.H. Target Gene Product Involves Cross-Regulation Between Wnt and NF-κB Pathways

Thyroid cancer-1 (TC1 or C8orf4) is a small protein present in vertebrates. TC1 is a novel endothelial inflammatory regulator enhancing NF-κB activity[60] that up-regulates heat-shock proteins.[61] TC1 is also a target gene of NF-κB signaling and up-regulates the Wnt/β-catenin pathway by relieving the antagonistic activity of Chibby (Cby), a nuclear β-catenin-associated antagonist of the Wnt/wingless pathway[62] for β-catenin-mediated transcription.[63] Cby is associated with inflammation and aggressive behavior in cancer with poor survival. Upon coexpression in mammalian cells, TC1 redistributes from the nucleolus to nuclear speckles, where it co-localizes with Cby. TC1 also up-regulates the expression of β-catenin target genes that are implicated in invasiveness and aggressive behavior of cancers, such as metalloproteinases, laminin gamma2, and others.[63]

The expression of leucine zipper tumor suppressor 2 (lzts2) is positively regulated by NF-κB activity in colon, liver, and breast cancer cells, whereas it is negatively regulated in glioma cells. Through lzts2, NF-κB negatively regulates the Wnt/β-catenin signaling pathway in colon, liver and breast cancer cells, whereas it has an opposite effect on this signaling pathway in glioblastoma. These findings indicate that NF-κB cross-regulates Wnt/β-catenin signaling via lzts2 in various human cancer cells.[64]

IV.I. Wnt-Regulated β-TrCP Mediates Cross-regulation of Wnt and NF-κB Pathways

1. β-TrCP Expression is Associated with Wnt and NF-κB Pathways

The ubiquitin/proteasome pathway is involved in the cross-regulation between Wnt and NF-κB by promoting ubiquitination of IκBα and β-catenin for their degradation. These molecular events lead to positive

and negative regulation of the NF-κB and Wnt pathways, respectively. β-TrCP, an E3 ubiquitin ligase receptor, is a component of the ubiquitin ligase complex targeting β-catenin and IκBα for proteasomal degradation by specifically recognizing a 19-amino-acid destruction motif in IκB and β-catenin.[65] With targeted disruption of the β-TrCP gene in knockout mice, IκB and β-catenin degradation can be prevented.[66]

2. β-TrCP Is a Target of the Wnt/β-Catenin Pathway and Up-regulates NF-κB and Down-regulates Wnt/β-Catenin Signaling

Several lines of evidence support a role for β-TrCP in the cross-regulation between Wnt/β-catenin and NF-κB signaling. A very important finding for the mechanism of the cross-regulation is that β-catenin/Tcf signaling elevates the expression of the β-*TrCP* mRNA and protein in a Tcf-dependent manner, which does not require β-TrCP transcription. Induction of β-TrCP expression by the β-catenin/Tcf pathway results in an accelerated degradation of the wild-type β-catenin, suggesting that a negative feedback loop may control the β-catenin/Tcf regulation. This signal also up-regulates NF-κB transactivation without affecting IκB kinase activity. Therefore, the maintenance of the β-TrCP level is important for coordination between β-catenin/Tcf and NF-κB signaling.[67] Endogenous β-TrCP1 expression is regulated through the conserved Wnt cascade. Up-regulation of Wnt1 results in the β-catenin-mediated activation of Tcf-4, leading to increased β-TrCP1 expression and NF-κB activity in vascular smooth muscle cells.[68] The relationship among β-TrCP, β-catenin, and NF-κB in colorectal cancer has shown that increased expression of β-TrCP1 is associated with the activation of both β-catenin and NF-κB, suggesting that integration of these signaling pathways by increased β-TrCP expression may contribute to an inhibition of apoptosis and tumor metastasis.[69,70]

3. Overexpression of CRD-BP Stabilizes β-TrCP mRNA

β-catenin also stabilizes the mRNA encoding for β-TrCP1, and identifies the RNA-binding protein CRD-BP (coding region determinant-binding protein) as a previously unknown target of the β-catenin/Tcf transcription factor.[71] CRD-BP binds to the coding region of β-TrCP1 mRNA. Overexpression of CRD-BP stabilizes β-TrCP1 mRNA and elevates β-TrCP1 levels in vitro and in vivo, resulting in the activation of the SCF (β-TrCP) E3 ubiquitin ligase, and in accelerated turnover of its substrates, including IκB and β-catenin, in colorectal cancer

cells.[71] High levels of CRD-BP are found in primary human colorectal tumors and malignant melanomas exhibiting active β-catenin/Tcf signaling, implicating CRD-BP induction in the up-regulation of β-TrCP1, in the activation of dimeric transcription factor NF-κB, and in the suppression of apoptosis in these cancers.[71,72]

IV.J. Epigenetic Modifications Medicate Cross-regulation Between Wnt and NF-κB Pathways

Post-translational modification of Tcf/Lef includes phosphorylation, acetylation, sumoylation, and ubiquitination/degradation.[7] CD44 overexpression and Wnt/β-catenin activation have been observed in colon cancer. The expression of CD44, a cross-membrane protein, and a receptor of hyaluronan (HA), is regulated by the Wnt pathway.[73] HA binding to CD44 up-regulates p300 expression and its acetyltransferase activity, which in turn promotes acetylation of β-catenin and NF-κB-p65, leading to activation of β-catenin-associated Tcf/Lef transcriptional co-activation and NF-κB-specific transcriptional up-regulation, respectively. This interaction can be reversed by activation of the NAD-dependent deacetylase sirtuin-1 (SIRT1) by resveratrol (a natural antioxidant). Resveratrol induces SIRT1-p300 association and acetyltransferase inactivation, leading to deacetylation of HA/CD44-induced β-catenin and NFκB-p65, inhibition of β-catenin-Tcf/Lef enhancer factor, and NF-κB-specific transcriptional activation.[74] The Wnt target gene product, CD44 triggers the post-translational modification and up-regulates Wnt/β-catenin and NF-κB signaling, as well as MDR and Bcl-xL gene expression, respectively. Through these modifications, breast cancer cells gain anti-apoptosis/survival benefit and chemotherapeutic resistance.[74]

IV.K. Cross-regulation Between Wnt/β-catenin and NF-κB Pathways Indicates the Link Between Chronic Inflammation and Tumorigenesis

Epithelia of the vertebrate intestinal tract characteristically maintain an inflammatory hyporesponsiveness toward the lumenal prokaryotic microflora. The identification of enteric organisms (nonvirulent *Salmonella* strains) whose direct interaction with model human epithelia attenuates synthesis of inflammatory effector molecules elicited by diverse proinflammatory stimuli has been reported. This immunosuppressive effect involves inhibition of the IκB/NF-κB pathway by blockade of IκBα degradation, which prevents subsequent nuclear

translocation of the active NF-κB dimer. These data suggest that prokaryotic determinants can be responsible for the unique tolerance of the gastrointestinal mucosa to proinflammatory stimuli.[75]

Salmonella-epithelial cell interactions are known to activate the proinflammatory NF-κB signaling pathway and have recently been found to also influence the β-catenin-signaling pathway. By using polarized epithelial cell models, the same bacteria-mediated effects were shown to be involved in the molecular cross-regulation between the NF-κB and the β-catenin signaling pathways. Convergence of these two pathways is a result of the direct interaction between the NF-κB p50 subunit and β-catenin.[76] PhoP(c), the avirulent derivative of a wild-type *Salmonella* strain, attenuates NF-κB activity and the expression of its target gene, IL-8, by stabilizing the association of β-catenin with NF-κB. These findings strongly suggest that the cross-regulation between the β-catenin and NF-κB pathways is an important regulator of intestinal inflammation.[76] Moreover, the same research group also found that AvrA, a bacterial effector existing in *Salmonella*, cross-regulates Wnt and NF-κB signaling pathways in colonic epithelial cell inflammation by deubiquitination, leading to increased β-catenin and decreased NF-κB activation.[77] On the other hand, β-catenin also plays an opposing role in the regulation of the NF-κB pathway. Wild-type *Salmonella* infection directly increases GSK-3β activity, which phosphorylates β-catenin, leading to its degradation, and further decreasing the physical association between NF-κB and β-catenin, which consequently increases NF-κB activity.[47] As mentioned previously, *Helicobacter*-infected k-19-wnt1 mouse stomach activates macrophages and causes β-catenin nuclear accumulation. This experimental model provides pivotal evidence that the macrophage-derived TNFα promotes Wnt/β-catenin signaling, which may influence gastrointestinal oncogenic potential. This also supports the observation that malignancy is frequently preceded by chronic inflammation in individuals harboring activating mutations in the *APC* or *CTNNB1* genes or enhanced activation of Wnt/β-catenin signaling.[37,38]

V. RECIPROCAL REGULATION OF WNT AND NF-κB PATHWAYS

V.A. Wnt Signaling Initiates Interdependent Regulation with NF-κB

Using hair follicle induction as a model system, the patterning of dermal Wnt signaling requires epithelial β-catenin activity. Wnt signal-

ing is absolutely required for NF-κB activation, and *Edar* is a direct Wnt target gene.[78] Wnt signaling is initially activated independently of EDA/EDAR/NF-κB activity in primary hair follicle primordia. However, Eda/Edar/NF-κB signaling is required to refine the pattern of Wnt activity and to maintain this activity at later stages of placode development. Maintenance of localized expression of Wnt10b and Wnt10a requires NF-κB signaling, provides a molecular explanation for the latter observation, and identifies Wnt10b as a direct NF-κB target. Moreover, DKK4, a Wnt/β-catenin signaling antagonist,[79] is a target gene of Eda/Edar.[80] NF-κB indirectly limits Wnt activity by activating DKK4, which in turn inhibits β-catenin signaling. These data reveal a complex interplay and interdependence of the Wnt and EDA/EDAR/NF-κB signaling pathways in the initiation and maintenance of primary hair follicle placodes.[78] These studies imply that NF-κB signaling may limit Wnt/β-catenin activity and refine the pattern of hair placode borders by establishing a DKK4-mediated negative-feedback regulation.

V.B. Interplay Between Wnt/β-Catenin and NF-κB Pathway Regulated by lzts2

The modulation of NF-κB activity shows a direct correlation with β-catenin/Tcf pathway in human adipose tissue (hASCs) and bone marrow (hBMSCs)-derived mesenchmal stem cells. Expression of lzts2, which represses β-catenin nuclear translocation and transcription activity, is positively regulated by NF-κB signaling.[81] Interestingly, down-regulation of lzts2 increases β-catenin and NF-κB activity in hASCs, increases the proliferation of hASCs and hBMSCs, and blocks the NF-κB-inhibitor-induced repressive effects on proliferation and Tcf promoter activation. Moreover, the activated NF-κB induced by the down-regulation of lzts2 is accompanied by increased β-TrCP expression and decreased IκB levels. The reciprocal cross-regulation of the β-catenin/Tcf pathway by NF-κB is mediated by lzts2 in hASCs.[81]

VI. CONCLUSIONS

Over the last 20 years since their identification, great progress has been made in understanding the complex role of the Wnt and NF-κB signaling pathways. Wnt and NF-κB signaling exert crucial roles in development, homeostasis, and pathogenesis by regulating the transcription of cell type-specific programs of Tcf and NF-κB target genes. However, the mechanisms and biological effects of the cross-regula-

tion of these two pathways remains an area of intense investigation. The studies of cross-regulation allow detailed analyses of Wnt and NF-κB signaling pathway interactions responsive to either Wnt or NF-κB signals.

Most of models provide evidence for mechanisms of cross-regulation between Wnt and NF-κB pathways either through β-catenin and NF-κB physical interaction or through target gene expression of the pathways for the convergence of the cross-regulation of both cell and animal models. With these notions, it is expected that dissection of the interdependent mechanisms regulating these pathways will demonstrate more insight into the cell biology of a signaling network crucial for development, homeostasis, and carcinogenesis.

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Inducible Nitric Oxide Synthase/ Cyclooxygenase-2 Pathway Interaction: A Good Molecular Target for Cancer Treatment

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ABSTRACT: An increase in the expression and activity of both inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) has been shown in several types of human tumors. A large body of evidence has demonstrated that these two enzymes are involved in tumor progression through several molecular mechanisms, such as promotion of tumor cell proliferation, inhibition of apoptosis, and stimulation of angiogenesis. iNOS and COX-2 share a number of similarities in terms of pathophysiological phenomena, and are often co-expressed in cancer tissues. The product of iNOS, nitric oxide (NO), has been demonstrated to modulate COX-2 expression and prostaglandin production in both inflammatory and tumor experimental models. Cyclic GMP and peroxynitrite, the coupling product of NO and O_2^-, appear to be the most important pathways by which NO may regulate COX-2 expression. We have recently shown that both NO- and COX-2-related angiogenesis are mediated by an increase in vascular endothelial growth factor (VEGF) production in colorectal cancer. We also provided evidence that NO can stimulate COX activity, and that its pro-angiogenic effect is mainly mediated by COX-2-related prostaglandin E2 (PGE_2) production. The purpose of this review is to summarize experimental data on the molecular mechanisms underlying iNOS-COX-2 cross-talk and investigate the pathophysiological significance of this interaction in cancer. Given the availability of highly selective inhibitors of both iNOS and COX-2, dual inhibition of these enzymes appears to be a promising therapeutic tool in the treatment of various types of human cancers possibly by producing a synergistic anti-tumor effect.

KEY WORDS: nitric oxide synthase, cyclooxygenase-2, prostaglandins, cancer, angiogenesis

I. INTRODUCTION

Nitric oxide (NO) and prostaglandins are two of the best-known mediators of the inflammatory process. NO is synthesized by a family of

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three NO synthase (NOS) isoenzymes that convert L-arginine into L-citrulline in the presence of molecular oxygen, yielding free NO.[1,2] The endothelial NOS and the neuronal NOS are Ca^{2+}- and calmodulin-dependent isoforms that are constitutively expressed in endothelial cells and neurons, respectively. They are responsible for low levels of NO production (in the picomolar to nanomolar range) for short periods. The third isoform, inducible NOS (iNOS), is Ca^{2+} independent and requires induction in response to cytokines and pro-inflammatory agents in essentially every cell type.[3] It can produce large quantities of NO (in the micromolar range) over an extended time (days to weeks), and induces the production of the second messenger, cyclic GMP (cGMP).[4] In inflamed tissue, iNOS is richly expressed by infiltrating and resident activated macrophages. The NO produced by activated macrophages may have important physiological benefits, such as antimicrobial and antiviral functions.[5] Inflammatory cytokines may also trigger iNOS expression by epithelial cells.[6] Chronic and sustained generation of epithelial cell-derived NO can be associated with direct reactions between NO and cellular constituents and the generation of reactive nitrogen species, with potentially detrimental consequences for the host.[7]

Cyclooxygenase (COX) is the enzyme responsible for the conversion of arachidonic acid to prostaglandins. There are two isoforms of COX: COX-1 and COX-2.[8] COX-1 is expressed constitutively in most tissues and appears to be responsible for the production of prostaglandins that control normal physiological functions, such as gastric cytoprotection, platelet aggregation, and regulation of renal blood flow. COX-2 is undetectable in most normal tissues, whereas it is rapidly induced by both growth factors and inflammatory stimuli, resulting in enhanced synthesis of prostaglandins, in particular prostaglandin E2 (PGE_2), in inflamed tissue.[9,10] In general, COX-2 expression under inductive stimuli follows the pattern of the so-called early genes, with a rapid increase after 2 to 4 h and a gradual diminution after 24 to 48 h.

iNOS shares significant features with COX-2 in terms of tissue distribution, regulatory function, and participation in pathophysiological phenomena. For example, their products, NO and PGE_2, are proven to provide a proliferative, survival, and angiogenic advantage for proliferating cells at inflammation sites. iNOS and COX-2 have been found to be frequently co-expressed within the same type of cells and under the same experimental circumstances, including inflammation,[11] coronary vasodilation,[12] cervical ripening during pregnancy,[13] cerebral ischemia,[14] and endotoxin-induced septic shock.[15] Moreover,

co-induction of COX-2 and NOS has been demonstrated in several cell lines after exposure to lipopolysaccharide (LPS)[16,17] and cytochines such as interleukin-1 (IL-1),[18] tumor necrosis factor-alpha (TNF-α),[19] and gamma-interferon.[17]

This evidence has led to the demonstration of an interrelationship between the activities of these two enzymes. Several studies have shown that NO can exert a stimulatory effect on COX-2 catalytic activity in various in vitro and in vivo systems.[20-26] However, the molecular mechanisms of this activation have not been elucidated yet. Salvemini et al.[20] have demonstrated that NO enhances COX-2 activity in the mouse macrophage cell line RAW264.7 through a mechanism independent of GMP, i.e., the downstream cyclic nucleotide effector of NO. Landino et al.[22] suggested that this effect is mediated by peroxynitrite ($OONO^-$), the coupling product of NO and O_2^-, possibly through its interaction with the heme group of COX. Another possibility is that lipid peroxidation initiated by peroxynitrite liberates arachidonic acid from the cell membrane, which in turn activates COX-2.[21] On the other hand, an inhibitory effect of NO on COX activation has also been reported.[27,28] The different modulatory actions of NO on COX induction have been attributed to its different concentrations in the cell microenvironment: large amounts of NO suppress prostaglandin production, while low levels activate COX. However, even the cell type investigated and/or the state of activation of the cells are likely to influence the type of response of COX-2 to NO stimuli.[18,29,30]

Interestingly, not only does NO modulate the activity of COX-2, but it has also been shown that NO can stimulate COX-2 protein and gene expression. The role of NO in inducing COX-2 expression has been assessed in a number of in vitro and in vivo experimental models of inflammation.[11,31] NO has been reported to modulate the signal-transduction cascades leading to COX-2 expression through the cGMP-dependent stimulation of tyrosine phosphatase activity[32] or activation of the c-Jun N-terminal kinase (JNK) and the p38 mitogen-activated protein kinase (MAPK).[33] A transcriptional regulation of COX-2 by NO has also been postulated. The human COX-2 promoter contains a number of sites for transcription factors that are potential targets for NO modulation.[31] Among these transcriptional factors, nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) appear important in the regulatory effects of NO on COX-2 gene expression.[34-36]

The recent demonstration of the NO-mediated up-regulation of COX-2 in colonic epithelial cells and in cholangiocytes may provide insight into the link between chronic inflammatory disorders and

carcinogenesis. Mei et al.[17] have demonstrated that exogenous NO increases the expression of COX-2 at both the mRNA and protein levels in the mouse colonic epithelial cell line YAMC. They have also suggested that transcriptional activation of the COX-2 gene may be a result of the NO-mediated accumulation of free soluble β-catenin in the cytoplasm and the formation of β-catenin/T-cell factor-lymphocyte enhancing factor (Tcf/Lef) DNA-binding complex in the nucleus. The NO-mediated stimulation of COX-2 mRNA and protein expression through a p38 MAPK- and JNK1/2-mediated pathway has also been demonstrated in the mouse cholangiocyte cell line 603B.[33] This study showed that the ability of iNOS to increase cholangiocyte growth is largely dependent on COX-2 induction and PGE_2 production. Collectively, these data suggest that chronic inflammatory disease such as ulcerative colitis or primary sclerosing cholangitis may predispose to colorectal cancer or cholangiocarcinoma, respectively, through the induction of both iNOS and COX-2.

A large body of evidence supports the hypothesis that the products of the iNOS and COX-2 pathways are involved in the regulation of several processes responsible for tumor growth. Both gene and protein overexpression of the two enzymes have been demonstrated in several experimental and human tumors.[7,37] Figure 1 summarizes the putative mechanisms underlying the carcinogenetic effect of iNOS and COX-2. iNOS activity has been associated with direct DNA damage,[38,39] stimulation of angiogenesis,[40-42] alteration of the apoptotic process,[43,44] induction of oncogene expression,[45,46] and activation of transcription factors.[34-36] COX-2 activity has been linked to the stimulation of angiogenesis[47,48] and cell proliferation,[49,50] the inhibition of apoptosis[51,52] and immune surveillance,[53] and the activation of matrix metalloproteinases.[54]

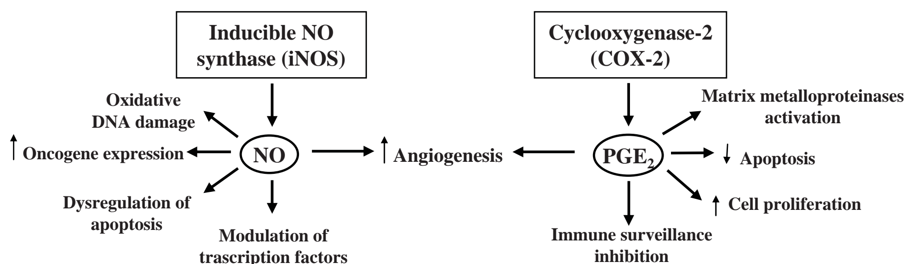

FIGURE 1. Role of NO and PGE_2 in carcinogenesis. NO and PGE_2 have been implicated in modulating several events involved in malignant transformation and cancer promotion/progression. A common and fundamental mechanism underlying the tumorigenic effect of NO and PGE_2 is the stimulation of angiogenesis.

iNOS and COX-2 have frequently been found to be co-induced within the same tumor cells. This finding supports the hypothesis of a causal relationship between the activity of the two enzymes even in tumor cells. The purpose of this review is to present the experimental data that provide evidence of the cross-talk between the iNOS and COX-2 pathways in some types of human tumors, the possible molecular mechanisms underlying the carcinogenetic effect of this interaction, and the potential of iNOS and COX-2 as molecular targets for cancer prevention and treatment.

II. INOS AND COX-2 INTERACTION IN PREMALIGNANCIES AND MALIGNANCIES

II.A. Colorectal Cancer

The possible independent role of either iNOS or COX-2 enzymes in colorectal tumor development and the possible underlying molecular carcinogenetic mechanisms in colorectal cancer have been extensively investigated.[55,56] Rao et al.[57] investigated the chemopreventive efficacy of the selective iNOS inhibitor SC-51, administered alone or in combination with the COX-2 selective inhibitor celecoxib, against azoxymethane (AOM)-induced formation of aberrant crypt foci (ACF) in rat colonic mucosa. They found that both iNOS and COX-2 activities are selectively inhibited in colonic mucosa by iNOS inhibitors after AOM treatment. Moreover, they showed that the administration of SC-51 plus celecoxib was more effective in inhibiting AOM-induced ACF formation than was administration of these agents individually. Collectively, these data suggest that suppression of iNOS activity may lead to the down-regulation of COX-2 activity in colonic mucosa. Furthermore, it is likely that an iNOS/COX-2 interaction is involved in early stages of colon carcinogenesis.

Overexpression of iNOS and COX-2 has been also correlated with advanced stages of disease in humans. However, few data exist regarding the possible cooperative effect of iNOS and COX-2 in promoting colorectal cancer progression. Bing et al.[58] compared iNOS activity, PGI_2 and thromboxane production, and COX-2 immunohistochemical expression in 11 colorectal cancer specimens and corresponding normal colonic mucosa, and found that the increase in iNOS activity and prostanoid production in tumors paralleled the increase in COX-2 expression, suggesting a possible co-regulation between iNOS and COX-2 in this type of tumor.

We have recently shown that iNOS and COX-2 are co-expressed within the same colorectal cancer cells, and that iNOS activity is significantly correlated with PGE_2 production in human tumor samples.[59] These data strongly indicate an interaction between iNOS and COX-2 activities in this type of tumor. In vitro data on the stimulatory effect of both endogenous and exogenous NO on COX-2 activity in the HCT116 and HT29 colon cancer cell lines confirmed our hypothesis. We demonstrated a co-induction of iNOS and COX-2 activities in response to epidermal growth factor (EGF) or LPS treatment in iNOS-positive/COX-2-negative HCT116 cells. The selective inhibition of iNOS activity by 1400W significantly reduced both LPS- and EGF-induced PGE_2 production. The stimulated production of PGE_2 was also inhibited by the COX-2 inhibitor celecoxib, suggesting that COX-2 is the main source of the endogenous NO-stimulated increase in PGE_2 production after LPS or EGF treatment. Administration of the NO donor sodium nitroprusside to iNOS-negative/COX-2-positive HT29 cells caused an increase in PGE_2 production that was reversed by celecoxib. Therefore, even exogenous NO seems to be correlated with increased COX-2 activity in colon cancer cells.

We also found that PGE_2 levels in tumor samples were significantly correlated with the degree of tumor angiogenesis assessed as intratumor microvessel density and vascular endothelial growth factor (VEGF) expression, whereas the products of the iNOS pathway did not appear to be correlated with any of the angiogenic markers that we investigated. These findings suggest a more direct involvement of COX-2, rather than iNOS, activity in the induction of tumor angiogenesis. This hypothesis was confirmed by our in vitro data. Co-induction of iNOS and COX-2 activities after LPS or EGF treatment had different effects on VEGF production in the HCT116 and HT29 cell lines. We did not find any increase in VEGF production in the iNOS-positive/COX-2-negative HCT116 cells, whereas the iNOS-negative/COX-2-positive HT29 cells showed a marked increase in VEGF levels. Moreover, the LPS- and EGF-stimulated production of VEGF in the HT29 cells was reversed by celecoxib. This finding suggests that the putative pro-angiogenic effect of NO in colorectal cancer is not direct, but is mainly mediated by COX-2 activity and thus PGE_2 production. This interaction is likely to produce a cooperative effect in stimulating VEGF-mediated tumor angiogenesis.

The ability of NO to induce COX-2 in colorectal cancer has also been demonstrated by Liu et al.[60] They found that the NO donor S-nitrosoglutathione increases both COX-2 protein expression and PGE_2 production in a dose- and time-dependent manner in HCA7, HT29,

and HCT116 human colon cancer cells. Recently, the same authors[61] have proposed an elegant model that elucidates the molecular mechanisms of NO-mediated induction of COX-2 in both non-transformed murine colonic epithelial cells and human colorectal cancer cells. NO treatment is known to cause the activation of matrix metalloproteinases, which lead to the degradation of E-cadherin. This effect contributes to the cytosolic accumulation of β-catenin and nuclear formation of the transcription complex between β-catenin and Tcf/Lcf. The investigators found that NO, through the above-mentioned β-catenin pathway, stimulates the expression of the transcription factor polyoma enhancer activator 3 (PEA3) and its binding with DNA. PEA3 has been shown to strongly stimulate COX-2 promoter activity, and thus to increase the transcription of the *COX-2* gene.

Altogether, these data clearly demonstrate a pivotal role of NO in stimulating COX-2 expression and activity in human colorectal cancer. It is likely that one of the most important mechanisms underlying the carcinogenetic effect of this cross-talk is PGE_2-mediated stimulation of VEGF synthesis, and thus tumor angiogenesis.

II.B. Esophageal and Gastric Cancer

iNOS and COX-2 have been demonstrated to be involved in Barrett's metaplasia, or columnar-lined esophagus, which arises in response to chronic reflux esophagitis and is associated with esophageal adenocarcinoma. Wilson et al.[62,63] examined endoscopic mucosal biopsies obtained from Barrett's esophagus and from control gastric tissues in the same patients. Surgical resection samples from adenocarcinomas arising in Barrett's mucosa and adjacent normal esophagus were also studied. An increase in mRNA expression of iNOS and COX-2 in 76% and 80% of Barrett's tissues, respectively, was found, and was significantly correlated with the expression of transforming growth factor-alpha (TGF-α)α. Up-regulation of both iNOS and COX-2 at the mRNA and protein levels was also found in esophageal adenocarcinomas arising in Barrett's mucosa compared with normal adjacent esophagus. These findings support the hypothesis that iNOS and COX-2 are involved early in Barrett's-associated neoplastic progression. It is likely that up-regulation of the two enzymes may be related to exposure of Barrett's epithelium to both the acid or acid plus bile salts as a consequence of duodenogastroesophageal reflux.

Van der Woude et al.[64] have provided some insights into the possible molecular mechanisms underlying the involvement of iNOS and COX-2 in the Barrett's metaplasia-dysplasia-carcinoma sequence.

They found that iNOS is highly expressed in Barrett's epithelium with intestinal metaplasia and in 50% of samples containing dysplasia, but not in Barrett's esophagus-associated adenocarcinoma. COX-2 immunostaining was negative in intestinal metaplasia and dysplasia, whereas it was present in most Barrett's esophagus-associated adenocarcinomas. They also evaluated the expression of some proteins involved in the apoptotic process, namely Bcl-2, Bax, and Bcl-xl, and demonstrated that the apoptotic balance in the transformation from intestinal metaplasia to adenocarcinoma switches to an anti-apoptotic phenotype because of increased Bcl-xl expression and decreased Bax expression. The investigators concluded that pharmacologic inhibition of COX-2 activity is unlikely to be effective in preventing Barrett's esophagus adenocarcinoma. Moreover, no clear correlation can be established between iNOS expression and activation of pro-apoptotic and anti-apoptotic genes.

In regard to gastric adenocarcinoma, Son et al.[65] investigated iNOS and COX-2 gene up-regulation in 23 tumor samples obtained from patients who had undergone gastrectomy, and found that COX-2 and iNOS mRNA were significantly higher in gastric cancer tissues than in the adjacent normal gastric mucosa. There was also a significant correlation between the level of iNOS and COX-2 mRNA in tumor samples. No significant association was found between mRNA levels and peritumoral gastric inflammation and status of *Helicobacter pylori* infection.

Rajnakova et al.[66] demonstrated a strong immunohistochemical expression of both iNOS and COX-2 in their 55 human gastric adenocarcinoma samples. This expression was significantly higher in large, advanced tumors than in small, early-stage ones. In the same tumors, the immunohistochemical accumulation of p53, which is an indicator for a loss of p53 tumor suppressor function, was found to correlate with iNOS and COX-2 expression. The investigators concluded that tumor-associated production of NO and prostaglandins may provide a selective growth advantage to tumor cells with mutant p53.

Van der Woude et al.[67] evaluated the expression of iNOS and COX-2 according to Lauren's gastric cancer classification into diffuse and intestinal adenocarcinoma types. It is known that gastric carcinomas of the diffuse type are associated with a poor prognosis compared with tumors of the intestinal type. Although all the tumor samples showed a high expression of both enzymes, no significant difference in the expression of either iNOS or COX-2 was found between the two types of tumors. The expression of Fas, Bcl-xl, Bcl-2, Bax, active caspase 3, and Ki-67 was investigated in the same tumor samples, but

no significant correlation was found between these apoptosis-related proteins and iNOS/COX-2 expression.

The pathogenesis of gastric lymphomas from mucosa-associated lymphoid tissue (MALT) has been demonstrated to be linked to chronic infection with *H. pylori*. It has also been shown that both iNOS and COX-2 are potentially involved in *H. pylori*-induced gastric mucosa alterations and development of this type of lymphoma.[68] Li et al.[69] demonstrated a high positive immunostaining rate for iNOS and COX-2 in their 32 gastric MALT lymphomas. In the same cases, COX-2 expression was significantly correlated with iNOS expression, tumor cell proliferative activity (assessed by Ki-67 labeling index), and p53 accumulation status. These findings suggest that iNOS and COX-2 may play a synergistic role in the evolution of *H. pylori*-associated gastritis to gastric MALT lymphoma.

II.C. Head and Neck Cancer

The possible interaction between iNOS and COX-2 pathways in head and neck squamous cancer (HNSC) was first investigated by Gallo et al.,[70] who found a significant correlation between NO and PGE_2 production and between iNOS and COX-2 mRNA/protein expression in human HNSC samples. Moreover, both iNOS and COX-2 expression were significantly correlated with lymph node metastases and the degree of tumor angiogenesis evaluated as microvessel density. Gallo et al.'s in vitro experiments on A-431 and SCC-9 HNSC cells showed that both endogenous (after iNOS induction by LPS and EGF) and exogenous NO increase PGE_2 production through the direct up-regulation of COX-2 mRNA and protein expression. These effects are likely to be mediated by a cGMP-dependent pathway, given that they are reversed by blocking guanylate cyclase.

The findings by Gallo et al. were confirmed by Park et al.,[71] who showed that the exposure of several HNSC cell lines to the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) increases COX-2 expression, resulting in increased PGE_2 synthesis. The up-regulation of COX-2 by NO has been demonstrated to be mediated via de novo synthesis of mRNA in experiments involving a transcription inhibitor and a COX-2 promoter activity assay. In addition, iNOS inhibitors have been found to down-regulate the NO-mediated overexpression of COX-2. The same induction of COX-2 has been obtained by adding exogenous cGMP, suggesting that NO-stimulated COX-2 up-regulation in HNSC cells is mediated by the activation of guanylate cyclase. Interestingly, it has also been shown that NO has no observable effect

on COX-2 in cancer cell lines with very low levels of COX-2 expression, while COX-2 protein is increased by NO and inhibited by iNOS inhibitors in cell lines with strong or moderate constitutive COX-2 expression. Therefore, Park et al. hypothesized that variations in increased COX-2 expression by NO may depend on whether signaling pathways inducing its expression are already active in cancer cells at the basal level.

Gallo et al.[72] reported the possibility of a close regulation of iNOS and COX-2 activities by the tumor suppressor gene *p53*. They found that their tumor samples expressing a mutated p53 protein released the highest levels of nitrite/nitrate and prostaglandins, suggesting a key role of p53 mutation in the up-regulation of both iNOS and COX-2. This hypothesis has been confirmed by in vitro studies, in which restoration of wild-type p53 in the A431 cancer line resulted in down-regulation of iNOS and COX-2 mRNA, protein expression, and products. These data suggest that iNOS and COX-2 are p53 target genes subjected to p53 repression, and might have important implications for the potential use of p53 gene therapy in head and neck squamous cancer.

II.D. Pancreatic Cancer

Inflammation has been identified as a significant factor in the development of pancreatic cancer. Both hereditary and sporadic forms of chronic pancreatitis may be associated with increased cancer risk.[73] iNOS and COX-2 have been demonstrated to be overexpressed in both chronic pancreatitis and pancreatic cancer, thereby providing a possible link between inflammation and tumor development. Immunohistochemical co-expression of iNOS and COX-2 has been shown in pancreatic adenocarcinoma by Kong et al.[74] COX-2 overexpression was positively correlated with high Ki-67 expression (i.e., tumor proliferation), while high iNOS expression was significantly associated with a high apoptotic index. Therefore, the activities of the two enzymes have been found to counteract each other. Although Kong et al. did not explain this finding, they suggested that COX-2 up-regulation in pancreatic cancer might be an antagonistic pathway of an iNOS-induced apoptotic system, and that NO-related apoptosis might be a result of various tumorigenic effects of NO, such as DNA damage, p53 mutation, and oxidation by nitrotyrosine. In this study, no correlation was found between iNOS/COX-2 expression and either patient prognosis or degree of tumor angiogenesis.

Franco et al.[75] investigated the expression of iNOS and COX-2 protein expression in pancreatic cancer by western-blot analysis,

and found a marked expression of both iNOS and COX-2 in tumor samples compared with paired normal pancreatic tissue. Moreover, co-expression of the two enzymes was present in all tumor samples. A moderate expression of COX-2 was found in the surrounding non-neoplastic tissue, suggesting the involvement of this enzyme in early tumor development via chronic inflammation.

Contrasting results were obtained by Kasper et al.,[76] who detected iNOS and COX-2 immunoreactivity in only 52.5% and 37.5%, respectively, of their 40 cases of pancreatic carcinomas. Moreover, they did not find any correlation between iNOS and COX-2 expression or between the expression of the two enzymes and the degree of tumor angiogenesis assessed as microvessel density.

Altogether, these findings suggest that the role of the NO/PGE_2 interaction and the underlying tumorigenic mechanisms in pancreatic cancer are not completely defined.

II.E. Other Types of Tumors

The possible opposite role of iNOS/COX-2 in ovarian tumors and tumor-associated macrophages was investigated by Klimp et al.,[77] who found an overexpression of both iNOS and COX-2 not only in malignant tumors (adenocarcinomas), but also in borderline and benign ovarian tumors (cystadenomas), demonstrating the involvement of both enzymes in the progression of ovarian tumorigenesis. On the contrary, only a small proportion of the tumor-associated macrophages were found to express iNOS and COX-2 (i.e., were in an activated state against tumor cell proliferation). Klimp et al. suggested that ovarian tumors can release mediators that can suppress iNOS/COX-2 expression in tumor-associated macrophages, and thus suppress their tumoricidal capacity.

Raspollini et al.[78] evaluated iNOS and COX-2 expression in 78 stage III G3 cases of ovarian cancer, and found that immunohistochemical positivity for the two enzymes was associated with a poor prognosis after surgical and chemotherapeutic treatments. Moreover, this study showed that both iNOS and COX-2 negative ovarian carcinomas are correlated with complete clinical response to first-line chemotherapy.

Co-expression of iNOS and COX-2 has been shown in 100 hepatitis C virus (HCV)-positive hepatocellular carcinoma samples obtained from patients who had undergone curative hepatectomy.[79] Although only COX-2 was found to be significantly correlated with intratumor microvessel density, combined negative tumor expression

of both iNOS and COX-2 provided a significant advantage to patient survival. The authors of that study concluded that iNOS and COX-2 overexpression might be caused by a secondary effect of cytokines produced in response to HCV infection or by the direct activation of the HCV core protein. Moreover, the impact of iNOS and COX-2 on the prognosis of patients with HCV-positive hepatocellular carcinoma might be attributable to modulation of angiogenesis by COX-2.

Marrogi et al.[80] demonstrated that the expression of iNOS and COX-2 is significantly correlated with both VEGF expression and microvessel density in non-small-cell lung cancer. These findings suggest that stimulation of angiogenesis is one of the most important mechanisms involved in iNOS- and COX-2 mediated carcinogenesis in this type of lung tumor.

The possible involvement of iNOS and COX-2 in the pathogenesis of lymphocytic thyroiditis and thyroid tumors has been investigated by Nose et al.,[81] who found a stepwise increase in immunoreactive expression of both iNOS and COX-2 in epithelial cells from lymphocytic thyroiditis and follicular adenoma to papillary carcinomas, well-differentiated and poorly differentiated types, and follicular carcinomas. Moreover, the levels of the two enzymes were significantly correlated in all cases of thyroid disease. These findings suggest that iNOS and COX-2 pathway interaction may be involved in the early phases of thyroid tumorigenesis, and may provide a possible link between inflammation and carcinogenesis.

Angiogenesis plays a key role in the development of astrocytic gliomas. Hara et al.[82] examined the immunohistochemical expression of COX-2, iNOS, and VEGF in 51 high-grade astrocytomas, including 31 glioblastomas (grade IV) and 20 anaplastic astrocytomas (grade III), 49 low-grade astrocytomas (grade II), and 43 reactive astrogliosis specimens. A stepwise increase of COX-2, iNOS, and VEGF expression was found from astrogliosis in low-grade through high-grade astrocytoma. Moreover, COX-2 expression was significantly correlated with iNOS, VEGF, and intratumor microvessel density, whereas iNOS expression was weakly associated with the degree of angiogenesis. Hara et al. concluded that iNOS/COX-2 interaction may contribute to astrocytic tumorigenesis by promoting new vessel formation.

III. CONCLUDING REMARKS AND FUTURE DIRECTIONS

As summarized in Table 1, iNOS and COX-2 pathways and their interaction seem to be potentially involved in the majority of human solid tumors. Although the nature of this cross-talk is complex, most evidence

TABLE 1. Interaction Between iNOS and COX-2 and Its Possible Tumorigenic Effect(s) in Premalignant and Malignant Conditions

Premalignancy and/or malignancy	Methods of iNOS and COX-2 investigation	Tumorigenic effect(s)	Reference(s)
Colonic aberrant crypt foci	Enzymatic activities	Not investigated	57
Colorectal cancer and cell lines	Enzymatic activities, mRNA and protein expression, immunostaining	Stimulation of angiogenesis	58–60
Barrett's esophagus and esophageal adenocarcinoma	mRNA and protein expression, immunostaining	Inhibition of apoptosis (?)	62, 64
Gastric cancer	mRNA expression, immunostaining	Association with p53 mutation	65–67
Gastric MALT lymphoma	Immunostaining	Increase in cell proliferation, association with p53 mutation	69
Head and neck cancer and cell lines	mRNA and protein expression, enzymatic activities, immunostaining	Stimulation of angiogenesis, association with p53 mutation	70–72
Pancreatic cancer	Protein expression, immunostaining	Increase in cell proliferation (?)	74, 75
Ovarian adenocarcinoma	Immunostaining	Not investigated	77, 78
Hepatocellular carcinoma	Immunostaining	Stimulation of angiogenesis	79
Lung carcinoma	Immunostaining	Stimulation of angiogenesis	80
Lymphocytic thyroiditis and thyroid tumors	Protein expression, immunostaining	Not investigated	81
Astrocytic gliomas	Immunostaining	Stimulation of angiogenesis	82

points to a stimulatory effect of NO on both COX-2 activity and expression. From the majority of the above reported studies, it can be inferred that iNOS and COX-2 products may represent a common final pathway controlling various tumorigenic mechanisms. Among these, stimulation of tumor angiogenesis appears to be the most frequently involved. However, the products of the iNOS pathway did not appear to directly correlate with specific angiogenic markers such as intratumor microvessel density or VEGF expression. This observation suggests a more direct link with COX-2 than with iNOS in the induction of angiogenesis, as well as the possibility that the potential pro-angiogenic role of NO is mainly mediated by inducing COX-2 activity and PGE_2 production. As a general mechanism, it might be hypothesized that the ability of iNOS to promote cancer cell growth is largely dependent on COX-2 induction. Figure 2 summarizes a possible model of signaling pathways for iNOS-mediated COX-2 induction in tumor cells.

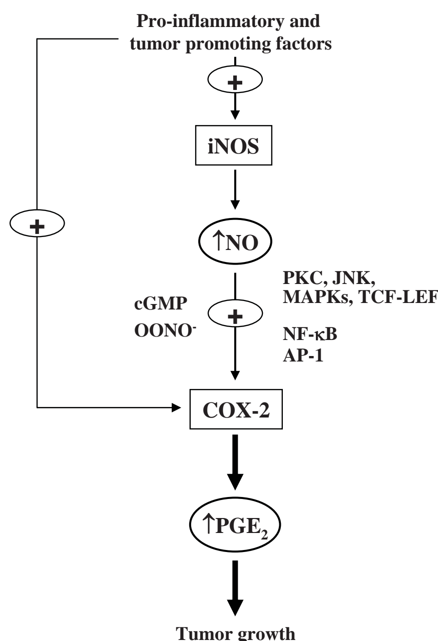

FIGURE 2. Proposed model of NO signaling in COX-2 activation and enhancement of tumor growth. Pro-inflammatory and tumor-promoting agents stimulate the activation of both iNOS and COX-2. iNOS produces NO, which enhances both the activity and expression (via a transductional and transcriptional regulation) of COX-2. COX-2 activation leads to the production of large amounts of tumor-promoting PGE_2. Therefore, dual inhibition of iNOS and COX-2 is an ideal strategy for cancer chemoprevention and therapy. AP-1, activator protein-1; JNK, Jun N terminal kinase C; TCF-LEF, T-cell factor lymphocyte enhancing factor; κPKC, protein kinase C; +, = stimulation; ↑, increase.

The selective inhibition of COX-2 in cancer has become one of the most actively investigated areas in molecular-targeted anti-tumor therapy. However, the majority of studies are limited to the development of selective inhibitors of COX-2 activity. It is likely that blocking COX-2 expression in cancer cells by inhibiting upstream stimulating factors such as NO may offer a more effective therapeutic solution than merely neutralizing the activity of existing COX-2 enzyme. Consistent with this concept, combination treatment with iNOS and COX-2 inhibitors may provide either a cooperative or a synergistic anti-tumor effect.

Both iNOS and COX-2 have been shown to be expressed in inflammatory disease (i.e., Barrett's esophagus, *H. pylori* gastritis, pancreatitis, ulcerative colitis, primary sclerosing cholangitis) and in the cancers arising from these diseases. The interrelationship between these two enzymes may provide a mechanistic link between hyper-inflammatory states and cancer susceptibility. In this regard, iNOS and COX-2 inhibition would appear to be the most proximal and optimal targets for chemoprevention of inflammation-related tumors.

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Broad-Spectrum Anti-Cancer Activity of O²-Arylated Diazeniumdiolates

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ABSTRACT: O^2-(2,4-Dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K) and O^2-{2,4-dinitro-5-[4-(*N*-methylamino)benzoyloxy]phenyl} 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (PABA/NO) are O^2-arylated diazeniumdiolates that have shown promising in vivo activity in a variety of rodent cancer models, including prostate cancer, leukemia, liver cancer, multiple myeloma, and ovarian cancer. This compound class was designed to be activated for anti-cancer effects by glutathione-*S*-transferase (GST)-induced release of cytotoxic nitric oxide (NO), but mechanistic studies have implicated a variety of pathways, some GST/NO-related, some not. Current work is focused on improving formulations and other drug development activities, as well as exploring possible new applications of these agents and their analogs. The selectivity of these drugs for attacking tumors while exhibiting little toxicity toward normal tissues suggests considerable promise for the treatment of various tumor types.

KEY WORDS: nitric oxide, JS-K, arylating agents, glutathione, PABA/NO

I. INTRODUCTION

This paper reviews published work about a compound class known as "arylated diazeniumdiolates," which are showing increasing promise as anti-cancer drugs.

I.A. The Starting Point

In comparing notes across the chemistry/biology interface some years ago with my University of Utah hematologist/oncologist colleague Paul Shami, he mentioned some pioneering results he and other colleagues had published earlier indicating that leukemia cells are substantially more sensitive to nitric oxide (NO) toxicity than most other mammalian cell types.[1] It turns out that NO is so crucial for so many normal life pro-

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cesses that evolution has provided us with mechanisms for protecting ourselves from its potential toxicity, much as we have evolved means to shield ourselves from another inherently toxic species that is essential for our well-being: oxygen. Apparently, leukemia cells have lost some of that protection against NO.

That being the case, we reasoned, it might be possible to administer an NO-releasing drug systemically in a leukemia patient, allowing it to distribute throughout the body but expecting it to selectively annihilate the sensitive leukemia cells without inflicting harm on other tissues and cell types.

It happened that my chemist colleagues Joe Saavedra and Aloka Srinivasan had earlier shown in a basic research effort that arylated diazeniumdiolates of general structure **1** could serve as caged NO sources whose doors could be opened by reaction with attacking nucleophiles such as isopropylamine and hydroxide ion to free structure **2**, an ion known to hydrolyze spontaneously to form NO (Fig. 1).[2] Given the success they reported in their first paper describing the fundamental chemistry of this compound type, it occurred to us that even stronger nucleophiles, such as glutathione (GSH) and other thiols that are abundant in the body, might easily provoke NO release in vivo, and thus compounds of structure **1** might be candidates for anti-leukemic drug discovery.

II. RESULTS AND DISCUSSION

Accordingly, Paul screened a library of Joe's arylated diazeniumdiolates for their in vitro cytotoxic activity toward two human leukemia cell lines, HL-60 and U937. He identified O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K) as the most active compound of the series. Paul then implanted HL-60 cells subcutaneously in the flanks of immune-compromised (non-obese diabetic severe combined immune deficient, or NOD-SCID) mice inca-

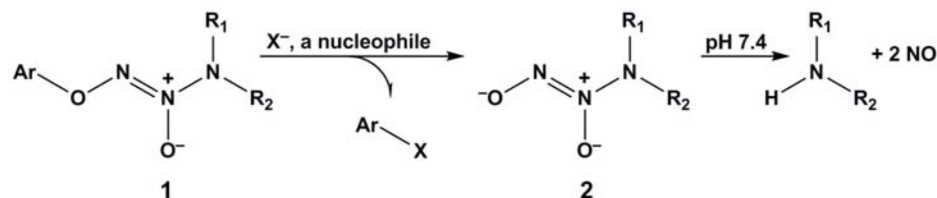

FIGURE 1. O^2-Arylated diazeniumdiolate 1 as a prodrug of NO. A nucleophile X^- displaces ionic diazeniumdiolate **2**, which then spontaneously releases up to two equivalents of NO at physiological pH.

pable of rejecting the xenografted cells, and charted the growth of the resulting tumor mass over time in animals receiving tail vein bolus injections of either JS-K or the vehicle in which the JS-K was dissolved. The JS-K dose he chose was the maximum that could be administered without inducing NO-mediated hypotension in these mice, 4 μmol/kg. This was given three times per week, and tumor volume was estimated by measuring the dimensions of the tumor implants every other day for the duration of the experiment. The results showed that JS-K cut the tumor growth rate in half, and histochemical examination of tumor explants revealed that the drug had induced substantial tumor necrosis as opposed to vehicle-treated control animals.[3] This initial set of experiments that Paul conducted was the first to demonstrate the anti-cancer activity of arylated diazeniumdiolates, and showed their potential for introduction to the clinic. Microarray analysis conducted later in collaboration with Jie Liu and Mike Waalkes of the National Cancer Institute showed the involvement of numerous pathways, including apoptosis and differentiation-related genes, acute phase protein genes, and genes related to angiogenesis.[4]

II.A. Solid Tumor Surprises

Very interestingly, quite similar results were observed in a prostate tumor cell line not known to be particularly sensitive to NO-induced toxicity. As with the leukemia line, Paul implanted PPC-1 human prostate cancer cells in the flanks of NOD-SCID mice and made similar observations as with the leukemia cells. Here, too, explants from JS-K-treated mice showed extensive tumor necrosis compared with vehicle-treated animals (Fig. 2).[3]

Meanwhile, Brian Carr of the University of Pittsburgh began studying the action of JS-K on human Hep3B hepatoma cells. Mechanistic studies conducted by Brian et al. implicated the mitogen-activated protein-kinase (MAPK) pathways in the apoptotic demise of these cells.[5] Subsequently, using an orthotopic syngeneic rat hepatoma model with JM-1 cells implanted in the liver of Fischer rats, Brian showed that JS-K inhibited the growth of liver cancer cells in vivo. Tumor growth inhibition was dose dependent.[6]

II.B. Multiple Myeloma and JS-K

Having noted Paul's successes in the leukemia model, Ken Anderson and Tanyel Kiziltepe of Harvard began a study of JS-K's potential as a therapy for multiple myeloma. Using a treatment protocol similar

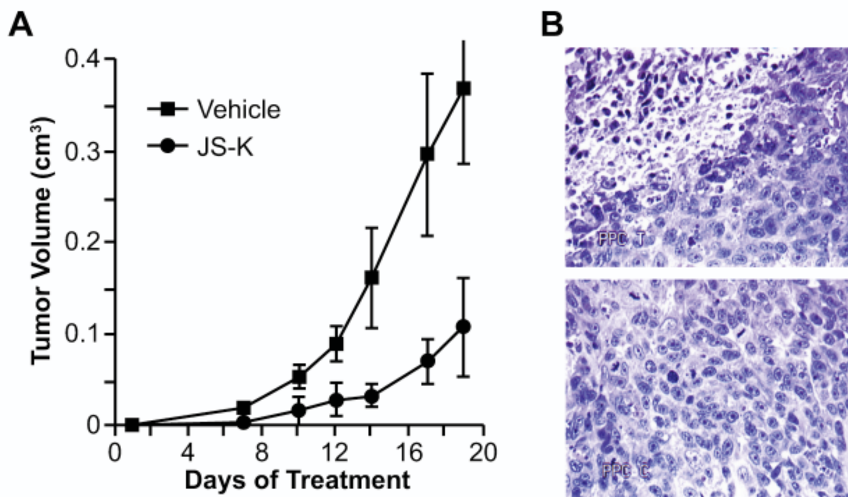

FIGURE 2. JS-K significantly slowed the rate of prostate xenograft growth (A) and induced necrosis in the remaining tumor mass (B). Note that the tumor tissue shown in the JS-K-treated section at the top shows more necrotic areas than the untreated control tissue section shown at the bottom. (Adapted from Shami et al., 2003.[3])

to the one Paul employed, Tanyel found an even more dramatic effect on the growth of OPM-1 multiple myeloma xenografts in mice, observing a growth delay of about 2.8-fold relative to control, as well as significant survival prolongation (Fig. 3). In vitro experiments showed activation of apoptosis, JS-K-induced DNA double-strand breaks, and elimination of the growth advantage imparted to myeloma cells by bone marrow stromal cells.[7]

II.C. Tumor-targeting Selectivity

A very important property of JS-K identified in the multiple myeloma study was the greater toxicity it showed toward tumor cells relative to normal human peripheral blood mononuclear cells (PBMCs). Tanyel reported IC_{50} (the concentration required to inhibit cell proliferation by 50%) values for primary patient isolates as well as therapy-resistant and -responsive cell lines in the 0.3 to 2.5 μM range. She also observed that, at a concentration of 2.5 μM, JS-K was not toxic to PBMCs. At a concentration of 5 μM, JS-K was toxic to over 20% of PBMCs. That concentration of JS-K was toxic to the majority of myeloma cells tested.[7]

Subsequently, Paul set up bone marrow transplant experiments in mice in collaboration with his colleague Thai Cao at the University

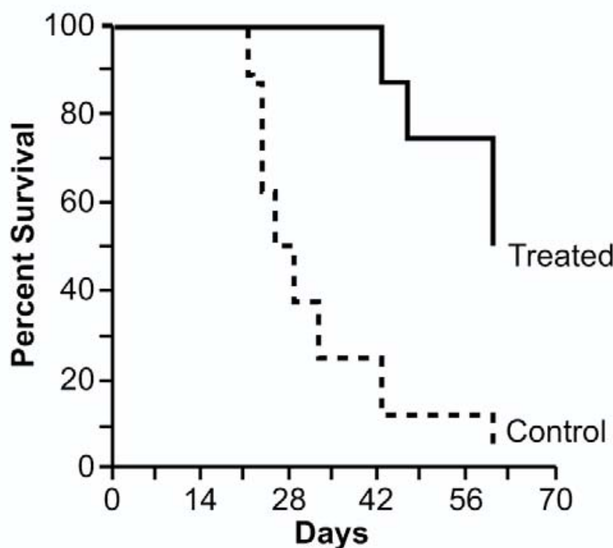

FIGURE 3. Intravenously administered JS-K significantly improved survival of mice bearing xenografts of OPM-1 multiple myeloma cells. (Adapted from Kiziltepe et al., 2007.[7] Copyright American Society of Hematology.)

of Utah. Paul and Thai showed that pretreatment of normal mouse hematopoietic stem cells with JS-K (at concentrations in the range of its IC_{50} for leukemia cells) did not affect survival or engraftment when these cells were used to rescue lethally irradiated mice.[8]

Meanwhile, Anna Maciag and Hari Chakrapani of NCI were following up results from the “NCI-60” tumor cell line screen,[6] suggesting activity on the part of JS-K against kidney cancer. They found IC_{50} values in the low micromolar range for three kidney cancer lines, but a value 20-fold less potent for the normal renal epithelial cell line HREpC (Fig. 4).[9]

It is perhaps relevant to note that the only toxic effect (other than those on the tumor) reported in any of the in vivo studies mentioned above was the hypotension expected for an NO-releasing drug at doses higher than the therapeutic doses. The mechanistic basis for this selectivity toward malignant cells as opposed to their normal counterparts is not yet known, but whatever its origin it is a most welcome attribute for an anti-cancer drug.

II.D. Synergies

Another desirable attribute in a drug candidate is the ability to augment the action of existing drugs. JS-K has been found to syner-

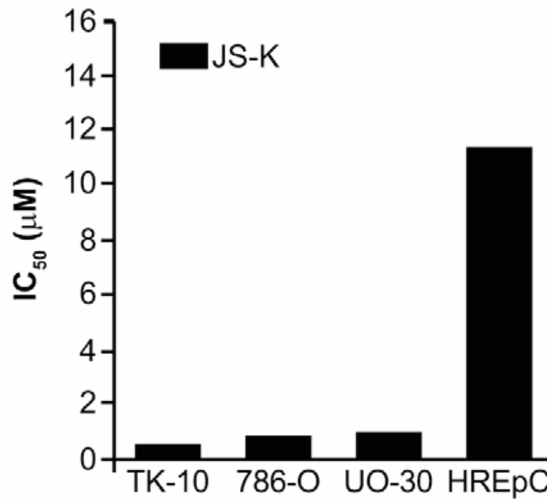

FIGURE 4. Selective inhibitory activity of JS-K against a panel of renal cancer cell lines (TK-10, 786-O, and UO-30) compared with the normal renal epithelial cell line HREpC. (Adapted from Chakrapani et al., 2008.[9])

gize with several. NCI's Liu-Waalkes team showed it to inhibit the multidrug resistance-related protein (MRP-1) efflux pump, allowing liver cancer cells resistant to the anti-leukemic agents cisplatin and sodium arsenite to accumulate the drugs in the cells and increase their toxicity.[10] Tanyel and colleagues reported JS-K's synergy with bortezomib in multiple myeloma cultures.[7] Paul found a synergistic anti-leukemic effect between cytarabine and JS-K, though his studies showed antagonism with other drugs.[11] Because prevailing clinical practice calls for new anti-cancer drugs to be introduced into human trials in combination with other agents, these early demonstrations of synergy could prove to be of great value in the future.

II.E. Anti-angiogenic Effects

In separate experiments, Gurmeet Kaur from NCI showed that JS-K inhibits different aspects of angiogenesis in vitro. These include inhibition of human umbilical vein endothelial cell (HUVEC) proliferation, migration, and cord formation. Furthermore, JS-K completely suppressed angiogenesis (induced by vascular endothelial growth factor or fibroblast growth factor) in the chick aortic ring assay. These in vitro observations were confirmed with the finding that JS-K inhibited tumor angiogenesis in vivo in Tanyel's multiple myeloma xenografts.[12]

II.F. Further Mechanistic Insights

In follow-up experiments, Paul showed that JS-K's anti-leukemic activity was dependent on NO release. He also showed that JS-K activates the intrinsic apoptosis pathway in leukemia cells directly through the loss of mitochondrial membrane potential and release of cytochrome *c* in the cytoplasm. Furthermore, Paul showed that JS-K activates the extrinsic apoptosis pathway indirectly.[3] Ana Tari, then of M.D. Anderson Cancer Center in Texas, studied JS-K's influence on the invasive properties of breast cancer cell lines, and reported that it inhibited matrigel invasion by breast cancer cells through the induction of tissue inhibitor of matrix metalloproteinase-2 (TIMP-2).[13] Ana also showed that JS-K induces autophagic cell death (cytoplasmic cell death or type II cell death) in a dose-dependent manner (concentrations higher than 1 μM) in all of the breast cancer cell lines tested.[14]

NCI's Yili Yang and Jirouta Kitagaki showed JS-K to inhibit ubiquitin ligase E1 in a variety of cell lines, reducing ubiquitylation and thus degradation of p53, and enhancing JS-K's ability to kill p53 wild-type cancer cells.[15]

While many of the effects seen in the various cell lines investigated so far in the JS-K studies are as expected for an NO-releasing drug, there is at least one other chemical pathway at work. Consider the activation mechanism that Joe and Aloka designed into the structure of this molecule, shown in Figure 5. In order to generate

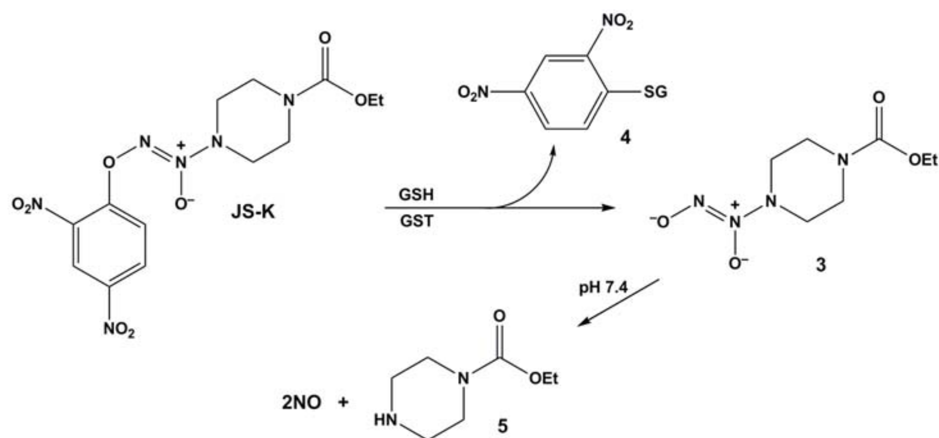

FIGURE 5. Metabolic activation pathway converting JS-K to carbamoylated piperazine 5, an arylated thiol moiety **4** (in this case that of GSH under catalysis by GST), and diazeniumdiolate ion **3**, which spontaneously hydrolyzes at physiological pH to produce up to two equivalents of NO.

NO, JS-K must react with cellular thiol groups or other nucleophilic species (shown here as GSH for the sake of illustration) to displace ionic diazeniumdiolate **3**, which then is freed to release NO spontaneously in the aqueous cellular environment. However, the attacking thiol group gets arylated to produce ionic diazeniumdiolate **4** in the process, effectively irreversibly. If the attacking nucleophile is a protein (PSH instead of GSH) whose function depends upon keeping its thiol group(s) free to maintain proper structure and reactivity, then that protein can be essentially taken out of action. Evidence that this pathway serves as a major factor in mediating JS-K's biological effects was seen in Paul's work with control compounds in HL-60 cells. The "pure" arylating agent 1-chloro-2,4-dinitrobenzene inhibited leukemia cell growth (IC_{50} 1.4 μM) and was somewhat better than spontaneously NO-generating ion **3** (IC_{50} 4 μM). Surprisingly, carbamoylated piperazine **5**, the carrier molecule that is left after the NO is released, was much more potent than expected (IC_{50} 8.6 μM), suggesting the possibility that a trans-carbamoylation pathway contributes to the mechanism of action. JS-K's submicromolar IC_{50} of 0.5 μM suggests that it combines all of these effects into a multifaceted chemical mechanism of action.[6]

Signaling pathways implicated in JS-K's activity are also clearly multifaceted, as summarized in Tables 1 and 2. Some would dismiss this richness of activity as the properties of a "dirty drug," one that hits too many targets to be worthy of further development. But it is increasingly clear that with the extent of genetic complexity observed in malignant cells there is great redundancy in the pathophysiologic mechanisms of cancer. Thus, with the notable exception of chronic myelogenous leukemia in the chronic phase, so-called "targeted therapies" have not held the promise that was hoped they would achieve. It may be that JS-K's multitude of molecular effects will prove to be a major advantage in our bench-to-bedside effort. It is also worth repeating that JS-K has so far shown little or no toxicity to the normal counterparts of two malignant cell types (leukemia and renal cancer) against which it was tested.

II.G. Lead Optimization

Having discovered JS-K in something of a random screening process, thought has been given to systematically modifying its structure to develop even more targeted anti-cancer action. Structural biologist Xinhua Ji of NCI knew that glutathione-*S*-transferase (GST) catalyzes NO release by JS-K, and that the π isoform of this enzyme is

TABLE 1. Genes Up-regulated by JS-K in HL-60 Human Leukemia Cells[4]

Apoptosis-related genes
caspase 3
caspase8
caspase9
BAX
TNF–α
Monocytic differentiation-related genes
CD14
CD11b
vimentin
Acute-phase genes
c-jun
EGR-1
Migration-related genes
TIMP-1
TIMP-2
TIMP-3
Anti-angiogenesis genes
thrombospondin-1
CD36

TABLE 2. Examples of Other Signaling Pathways Affected, Including Some That Are Cell Type-Dependent

ER-negative breast cancer cells	Up-regulated TIMP-2; induction of LC3-II and autophagy, but not apoptosis[13]
Hep 3B	Phosphorylation of ERK, JNK, AP1, p38[5]
Retinal pigment epithelial cells	Inhibition of p53 ubiquitination by inhibiting E1 and Hdm2[15]

overexpressed in many cancers. He was also intimately familiar with the active site characteristics of the three principal isoforms of GST (α, μ, and π) that are expressed to different extents in leukemia cells isolated from patients.[16] Kinetic studies have shown JS-K to be metabolized 100-fold more efficiently by α relative to π.[3] Reasoning that a reversal of this selectivity ratio would greatly increase the extent of cytolytic metabolism in the π-overexpressing cancer cells, Xinhua modeled the accommodation of JS-K in the active sites of each. Based on this structure-based drug design exercise, he suggested that altering the JS-K molecule as shown in (Fig. 6) would accomplish that goal.[17] Shrinking the size of the amino group to which the diazenium-

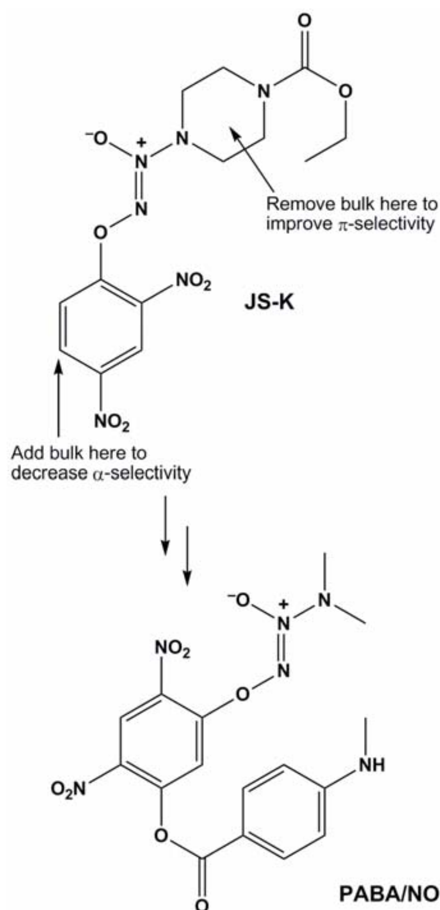

FIGURE 6. Results of molecular modeling experiments leading to the design of PABA/NO as a potential GSTπ-selective improvement over JS-K. (Reproduced from Saavedra et al., 2006.[17])

diolate group is attached should ease the accommodation in the π active site, while increasing the two-dimensional steric bulk of the aryl group should make metabolism by α more difficult. In response, Joe prepared PABA/NO, whose structure is shown in Figure 6. Kinetic studies showed this compound to be metabolized by π and α at comparable rates, an improvement of about 100-fold in the π/α ratio.[17]

II.H. In Vivo Activity Rivaling That of Cisplatin

PABA/NO has been studied in some detail by Ken Tew and Danyelle Townsend, currently of the University of South Carolina. They

first established that its mechanism of toxic action is GSH-, GSTπ-, and MRP-1-dependent. They then performed an in vivo experiment in mice, establishing human ovarian cancer xenografts and studying PABA/NO's effect on tumor growth rate. Here, too, the arylated diazeniumdiolate greatly retarded growth of the tumor mass relative to the vehicle control. Very significantly, PABA/NO's potency was comparable to that of the positive control cisplatin, which is used clinically for treating ovarian cancer.[18]

II.I. Current Problems and Future Plans

Our next goals in the effort to move JS-K and its analogs through the drug-development process and into the clinic are to increase the compounds' stability in the bloodstream and to increase their efficacy. To address these goals, we are examining a variety of formulations designed to shield the molecule from GSH and other nucleophiles encountered in the circulatory system, thereby slowing drug activation. Importantly, such a formulation should be scalable to clinical quantities and should be by itself safe. Paul has now developed such a formulation using nanoscale micelles. We are also continuing lead optimization studies with the hope that systematically fine-tuning the structure will lead to further improvements in the drugs' pharmacokinetics and, eventually, to the needed cures.

II.J. A Path Toward Clinical Development

Our goals cannot be achieved by one research group working in isolation, and I would like to close by acknowledging the essential contribution of all of my gifted collaborators (the "JS-K consortium") who have served as coauthors in the published works cited in the references section of my paper. Other invaluable collaborators are in the process of publishing newer work that will greatly advance the effort. In view of the enormous resources required to bring a new anti-cancer agent to the clinic, a commercialization effort is necessary. For this purpose, Paul and his colleague Thomas Kennedy from the University of Utah have founded JSK Therapeutics, Inc. JSKT has executed the necessary licensing agreements with the University of Utah and is leading the preclinical development of JS-K in preparation for an Investigational New Drug filing with the US Food and Drug Administration. JSKT has secured initial seed funding and is seeking further funding in order to start a phase I clinical trials program in about two years. Consequently, through the work of the multiple members of

the "JS-K consortium" and a serious commercialization effort, it will hopefully be possible to bring to the clinic a new class of potent anti-cancer agents.

ACKNOWLEDGEMENT

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Inhibition of Snail-induced Epithelial-Mesenchymal Transition and Induction of the Tumor Metastasis Suppressor Gene Raf-1 Kinase Inhibitor Protein (RKIP) by DETANONOate

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ABSTRACT: Tumor metastasis initiates through the epithelial to mesenchymal transition (EMT) process. Unraveling the underlying molecular mechanisms of EMT should identify novel targets for therapeutic intervention. Nitric oxide (NO) sensitizes resistant tumors to apoptosis through inhibition of the constitutively activated nuclear factor (NF)-κB signaling. Since NF-κB hyperactivation is associated with tumor metastasis via regulation of EMT, we hypothesized that NF-κB inhibition by NO should suppress EMT. We demonstrate that treatment of the metastatic human prostate cancer cell lines DU145 and PC-3 with (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate) inhibits significantly the constitutive NF-κB activity through p50 S-nitrosylation and the expression of the mesenchymal markers vimentin and fibronectin, where it augments the expression of the epithelial markers E-cadherin and cytokeratin 18. Also, the NO-treated cells had decreased migratory and invasive properties. We further show that NO-mediated NF-κB inhibition results in downstream inhibition of its transcriptional target, Snail (SNAI1), a known EMT inducer. Snail inhibition, in turn, triggers the induction of the metastasis suppressor gene products Raf-1 kinase inhibitor protein (RKIP) and E-cadherin, whose transcriptions are negatively regulated by Snail. Thus, RKIP induction further suppresses NF-κB and consequently inhibits EMT. The above findings were corroborated by cell transfections with Snail siRNA and RKIP overexpression vectors and were validated in vivo in mice bearing PC-3 xenografts that were treated with DETANONOate. These findings establish for the first time the role of NO in the inhibition of EMT via dysregulation of the NF-κB-Snail-RKIP circuitry, and suggest the potential therapeutic application of NO donors in the regulation of metastasis.

KEY WORDS: prostate cancer, metastasis, NF-κB, Snail, Raf-1 kinase inhibitor protein, RKIP, nitric oxide

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I. INTRODUCTION

Metastatic disease is the primary cause of death for most cancer patients. The available conventional approaches for metastasis management, chemotherapy, radiation, biological and hormonal therapies, such as surgery, cryosurgery, or a combination of these, unfortunately fail to control metastasis in most cases. Therefore, treatment of metastasis remains a major problem in cancer and hence the urgent need to identify targets for intervention.

Metastasis is the spread of tumor cells from a primary site (carcinoma in situ) into the surrounding tissues (micro-metastasis) or into distant organs (distant metastasis) through the bloodstream. One of the hallmarks of early metastasis is the loss of the epithelial phenotype of the primary tumor cells and the acquisition of a mesenchymal phenotype with invasive and migratory properties, a process known as the epithelial to mesenchymal transition (EMT). EMT allows the escape of epithelial cells and their migration to different locations via the transcriptional repression and delocalization of proteins participating in cell-cell junctions (e.g., cadherins), resulting in the reduction of cell-cell adherence.[1-3]

A number of genes participating in the establishment of tumor development by promoting cell growth, survival, and genomic instability are also involved in the initiation of metastasis, progression, and virulence functions. These functions are supported by dysregulated survival pathways, such as the nuclear factor (NF)-κB, phosphatidylinositol-3-kinase (PI3K), Ras/mitogen-activated protein kinase (MAPK), and their downstream transcriptional targets, or the lack of function of tumor and metastasis suppressor genes such as *PTEN* (phosphatase/tensin homolog deleted on chromosome 10), *TP53I* (tumor protein 53 inducible), or *RKIP* (Raf-1 kinase inhibitor protein). These contribute not only in tumor survival but also in promoting invasion, angiogenesis, bone marrow mobilization, and tumor infiltration to the circulation (metastasis initiation functions) as well as in tumor extravasation and reinitiation (metastasis progression functions), leading finally to organ-specific tumor colonization (metastasis virulence functions).[4-6]

Gene profiling of EMT has identified families of genes with well-characterized "EMT-inducer" functions such as the Snail (*SNAI1*) superfamily of transcription factors.[7] Snail and other EMT-inducer genes such as *Twist* and *SIP1* function as suppressors of the epithelial phenotype by down-regulating directly or indirectly the expression of the epithelial markers such as E-cadherin and α- and γ-catenins.[8]

In contrast, the above gene products promote the expression of genes that participate in the mesenchymal phenotype, such as fibronectin, vimentin, and E-cadherin.[9,10] Both Snail and Twist seem to be under the transcriptional regulation of the NF-κB and the MAPK pathways, suggesting that their expression is tightly controlled by the activation status of these pathways.[11-13]

The objective of the present review is to discuss molecular means by which the EMT phenotype is inhibited and therefore metastasis might be suppressed. Because the constitutive activation of the NF-κB pathway in tumors is involved in the regulation of the initiation of EMT, agents that are able to inhibit NF-κB and/or downstream NF-κB-regulated gene products should result in the inhibition of EMT. Thus, we hypothesized that nitric oxide (NO) donors may suppress the EMT phenotype via inhibition of NF-κB activity.

II. NO-MEDIATED INHIBITION OF EMT VIA INHIBITION NF-κB ACTIVITY

There is a broad spectrum of agents referred to in the literature as potential NF-κB inhibitors; these include proteasome inhibitors, specific synthetic chemical compounds such as the dehydroxymethylepoxyquinomicin (DHMEQ) and Bay-11-7082, and high concentrations of NO released by NO donors.[14-16] (Z)-1-[N-(2-aminoethyl)-N-(2-ammon ioethyl)amino]diazen-1-ium-1,2-diolate (DETANONOate) is an NO donor that belongs to the family of diazeniumdiolates (formerly called NONOates). It spontaneously dissociates in a pH-dependent, first-order process with a half-life of 20 h and 56 h at 37°C and 22°C to 25°C, pH 7.4, respectively, to liberate 2 moles of NO per mole of the parent compound. DETANONOate decomposition is not catalyzed by thiols or biological tissue unless specifically designed to be, because the NO release follows simple first-order kinetics.[17] A major characteristic of DETANONOate is that the rate of NO release can be accurately predicted. This can be achieved via specific modification of the NONOate structure, which can stabilize the drug in solution and potentially engender a selective NO release in different organs, vascular beds, or specific cell types.[18-20]

We tested our hypothesis that NO inhibits EMT via inhibition of NF-κB in the highly metastatic prostate tumor cell lines DU145 and PC-3, both of which are characterized by constitutive activation of NF-κB, and the non-metastatic prostate tumor cell line LNCaP. The inhibition of NF-κB by DETANONOate was confirmed at multiple levels. DETANONOate, at concentrations ranging between 500 and

1000 μM, was able to inhibit the NF-κB promoter and DNA-binding activities, as assessed by a reporter system and an electrophoretic mobility shift assay (EMSA), respectively. DETANONOate also promoted the S-nitrosylation of the p50 NF-κB subunit, resulting in further inhibition of NF-κB function as a transcription factor.

Treatment of DU145 and PC-3 cells with 1000 μM of DETANONOate for 4 or 12 h resulted in a significant reduction of the high baseline levels of fibronectin and vimentin, whereas it increased the almost absent levels of E-cadherin, as assessed by western-blot analysis. These results were also confirmed by immunofluorescence studies. In addition, the invasive properties of the above cells were significantly attenuated (>5-fold) after cell treatment with DETANONOate in concentrations ranging from 500 to 1000 μM, as assessed by an in vitro invasion monitoring assay.

Overall, these findings indicate that DETANONOate inhibits both NF-κB and the expression of mesenchymal gene markers, whereas it induces the reappearance of epithelial gene products. These events result in the reduction of the invasive properties of metastatic tumor cells via inhibition of EMT.

III. ROLE OF THE NF-κB/SNAIL/RKIP LOOP IN THE NO-MEDIATED INHIBITION OF EMT

In our effort to understand the underlying molecular mechanism of DETANONOate-mediated inhibition of EMT, we hypothesized that DETANONOate may regulate metastasis via modification of NF-κB-regulated gene products involved in EMT induction or suppression. The following questions were addressed. First, does DETANONOate down-regulate the expression of the transcription factor Snail? Snail was selected as a candidate DETANONOate target because of its transcriptional regulation by NF-κB and its role as a crucial EMT-inducer.[9] Because DETANONOate inhibits NF-κB, we hypothesized that DETANONOate also inhibits Snail downstream of NF-κB, resulting in inhibition of EMT. Second, does DETANONOate up-regulate the expression of the metastasis suppressor gene product Raf-1 kinase inhibitor protein (RKIP)? RKIP has been shown to inhibit NF-κB and metastasis and to be under the transcriptional repression of Snail.[12,13] If DETANONOate down-regulates Snail via NF-κB inhibition, we hypothesized that RKIP would be de-repressed by DETANONOate, resulting in inhibition of EMT. Third, does DHMEQ, a specific NF-κB inhibitor,[16] mimic DETANONOate in terms of Snail suppression and RKIP induction and reversal of EMT? Fourth, could our in vitro

findings on DETANONOate-mediated EMT inhibition be validated in vivo in mice bearing PC-3 xenografts?

III.A. DETANONOate Suppresses Snail Expression Through the Inhibition of NF-κB Activity

Snail is a member of the Snail superfamily of zinc-finger transcription factors.[9] Snail expression is crucial in embryonic development, neural differentiation, cell division, and cell survival. Snail has also an active role in the acquisition of invasive and migratory properties during tumor progression by triggering metastasis induction via direct transcriptional repression of metastasis suppressor genes such as E-cadherin and RKIP, or indirectly via modulation of gene products that determine the mesenchymal metastatic cell phenotype.[8,21,22] Snail is overexpressed in several tumor types with high metastatic potential,[9,23,24] and its expression is significantly reduced after NF-κB inhibition, suggesting that it is under the regulation of NF-κB.[12,13]

DU145 and PC-3 cell treatment with 1000 μM of DETANONOate resulted in a significant reduction of Snail expression. To examine the direct role of Snail in the induction of the EMT phenotype, Snail expression was knocked down in DU145 cells by small interfering RNA (siRNA). Snail silencing mimicked DETANONOate in terms of inhibition of the mesenchymal markers vimentin and fibronectin and up-regulation of the epithelial gene products E-cadherin and cytokeratin 18, resulting in the reversal of the EMT phenotype. The above findings were also corroborated by immunofluorescence studies. We further examined whether the EMT phenotype could be induced in the non-metastatic prostate tumor cell line LNCaP by ectopic expression of Snail. Overexpression of Snail in LNCaP by a cytomegalovirus (CMV)-triggered expression vector resulted in reversal of the epithelial cell phenotype by inhibiting the expression of E-cadherin and cytokeratin 18 and inducing the expression of vimentin and fibronectin.

Overall, the above findings demonstrate that DETANONOate down-regulates Snail expression, which has a direct role in EMT induction because its silencing reverses the EMT phenotype and its overexpression induces EMT.

III.B. DETANONOate Induces RKIP Expression Through the Inhibition of Snail Expression

RKIP is a member of the phosphatidyl ethanolamine-binding protein family. RKIP was initially identified as a cytosolic protein with roles

in reproduction, neurophysiology, and lipid metabolism.[25] More recently, RKIP has been shown to function as a specific inhibitor of both the Mitogen Activated Protein Kinases (MAPK) and NF-κB signaling survival pathways via its direct physical association with Raf-1 and the upstream activators of the canonical and non-canonical NF-κB pathways transforming growth factor-beta-activated kinase 1 (TAK1) and NF-kappa-B-inducing kinase (NIK), respectively.[26,27] RKIP also regulates G-protein-coupled receptors, and its overexpression modulates the apoptotic pathways resulting in reversal of tumor chemo- and immunoresistance.[28-30] RKIP is significantly repressed in several tumors and almost absent in metastatic cancers, while its ectopic expression has been shown to inhibit metastasis demonstrating its role as a metastasis suppressor.[31,32] Because RKIP transcription is directly repressed by Snail,[22] we hypothesized that DETANONOate-mediated Snail inhibition could result downstream in RKIP induction.

Cell treatment with DETANONOate resulted in early induction of RKIP protein expression, concomitant with Snail repression and inhibition of the mesenchymal cell phenotype. The direct role of RKIP induction in the DETANONONOate-mediated EMT inhibition was examined by ectopic expression of RKIP in DU145 cells using a CMV-triggered expression vector. Cells overexpressing RKIP showed decreased Snail expression, most likely through RKIP-mediated NF-κB inhibition, as well as suppressed expression of vimentin and fibronectin and increased levels of E-cadherin and cytokeratin 18. The above findings were corroborated by immunofluorescence analysis.

Overall, the above findings demonstrate that DETANONOate induces the expression of the metastasis suppressor gene product RKIP via inhibition of Snail, and that the overexpression of RKIP inhibits the mesenchymal cell phenotype and up-regulates epithelial markers. In addition, RKIP and Snail levels are inversely correlated, and their ratios seem to be involved in the regulation of the EMT phenotype.

III.C. DHMEQ Mimics DETANONOate in Inducing Snail Suppression and RKIP Induction and Reverses the EMT Phenotype

A crucial issue addressed in our study was the interrelationship among the NF-κB, Snail, and RKIP gene products shown above to be modified by DETANONOate. Specifically, we examined whether DETANONOate-induced repression of Snail is due in part to DETANONOate-mediated inhibition of NF-κB activity and whether a specific NF-κB inhibitor, DHMEQ,[16] mimics DETANONOate-induced

repression of Snail, induction of RKIP, and reversal of the EMT phenotype. Concomitant with the effect of DETANONOate on the expression profiles of Snail and RKIP, DU145 cell treatment with DHMEQ resulted in Snail suppression and RKIP induction. In addition, DHMEQ treatment was able to reverse the EMT phenotype of DU145 cells by inhibiting vimentin and fibronectin and up-regulating E-cadherin expression. These findings suggested that it is the regulation by DETANONONOate of the NF-κB-Snail-RKIP loop that results in the inhibition of EMT.

III.D. Validation of DETANONOate-Mediated Reversal of EMT Phenotype in Mice Bearing PC-3 Xenografts

To further validate our in vitro findings on DETANONONOate-mediated inhibition of the EMT phenotype in vivo, we used SCID mice bearing PC-3 xenografts that were treated with DETANONOate or phosphate-buffered saline as a control.[33] PC-3 cells (1×10^6) were administrated to mice by subcutaneous injection and left to grow for 5 weeks. DETANONOate was administered intratumorally at a concentration of 0.4 mg/kg every 2 d for 12 d. Tumor biopsies were harvested at week 9 post-tumor cell injection and analyzed by immunohistochemistry for RKIP, Snail, E-cadherin, vimentin, and fibronectin expression. The findings revealed that the biopsies derived from DETANONONOate-treated mice had a significant reduction of Snail, vimentin, and fibronectin expression, while the expressions of RKIP and E-cadherin were elevated compared with control mice (Huerta-Yepez et al., unpublished data). The above findings constitute an in vivo validation of our in vitro observations on the reversal of the mesenchymal metastatic phenotype of PC-3 cells by DETANONONOate.

IV. CONCLUDING REMARKS, IMPLICATIONS, AND FUTURE PROSPECTIVES

The present study demonstrates for the first time the interference of DETANONOate with the NF-κB/Snail/RKIP circuitry in the inhibition of EMT. The sequence of events taking place in the proposed mechanism of DETANONONOate-mediated EMT inhibition in metastatic tumor cells begins with the DETANONOate-induced inhibition of the NF-κB survival pathway and its downstream transcriptional target, Snail. This results in the subsequent down-regulation of the expression of EMT-promoting mesenchymal gene products such as fibronectin and vimentin. In contrast, Snail suppression de-represses

the transcription of the metastasis-suppressor gene products RKIP and E-cadherin. RKIP induction further inhibits NF-κB activation, thus attenuating the downstream activation of the NF-κB-regulated pro-metastatic gene products (Fig. 1). DETANONOate-mediated NF-κB, RKIP, and Snail modifications have a direct role in the regulation of EMT, as shown by specific gene knockdown and overxpression assays. Thus, the tight interference of DETANONONOate with the NF-κB/Snail/RKIP loop serves as a mechanistic model of DETANONOate-mediated regulation of EMT.

Our current and previous findings with DETANONOate demonstrate its significant clinical relevance in cancer treatment. The clinical relevance of inhibiting Snail and restoring RKIP expression by DETANONOate might correlate with a favorable clinical outcome when accompanied by inhibition of EMT and metastasis. In addition, RKIP induction by NO donors may improve the efficacy of anti-tumor therapies, especially if they are combined with conventional immuno- and/or or chemotherapeutics and the host immune surveillance against cancer.

Our future plans include further investigations of the molecu-

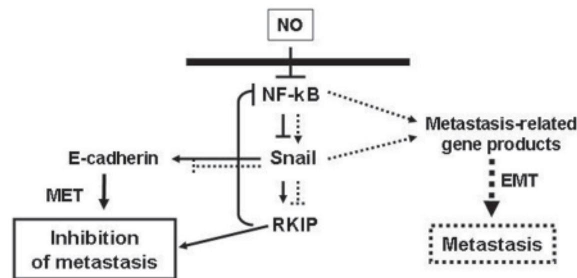

FIGURE 1. NO inhibits tumor cell metastasis via regulation of the NF-κB/Snail/RKIP loop. NO mediates its biological effects mainly by inhibiting the NF-κB pathway, and consequently, the expression of NF-κB-regulated gene products. The Snail transcription factor, an essential initiator of EMT, is under the positive regulation of NF-κB and inhibits the expression of metastasis suppressor genes such as *RKIP* and *E-cadherin*, while it induces directly or indirectly the expression of mesenchymal markers, resulting in the acquisition of a metastatic phenotype by the tumor cells. We show that, in addition to direct NO-induced NF-κB inhibition, NF-κB could also be inhibited by NO-mediated RKIP induction, resulting in the modulation of tumor cell metastatic potential. RKIP induction by NO may result from down-regulation of its transcriptional repressor Snail via inhibition of its upstream activator NF-κB by NO (feedback loop). The NO-mediated regulation of the NF-κB/Snail/RKIP loop via the above mechanism results in reversal of the mesenchymal cell phenotype (mesenchymal to epithelial transition or MET) and inhibition of the migratory and invasive properties of the tumor cells. Solid lines correspond to the NO-mediated effects on the indicated gene products; dotted lines correspond to the constitutive basal levels in tumor cells in the absence of NO.

lar mechanism of DETANONOate-induced down-regulation of EMT (used alone or in combination with chemo- or immunotherapy) in in vivo prostate tumor metastatic models. Special focus will be given to the effects of Snail-knockdown or RKIP overexpression in the metastatic behavior of the tumor cells in the above in vivo models. Finally, we will explore NO donors other than DETATONOate with more potent EMT-inhibitory therapeutics.

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