



Published in final edited form as:

J Immunother. 2010 May ; 33(4): 335–351. doi:10.1097/CJI.0b013e3181d32e74.

Cancer and Inflammation: Promise for Biological Therapy

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Financial Disclosure: MC, Y-CC, GC, LMC, SD, EME-O, MK, MTL, JTM, EP, ES, GT, EW have declared there are no financial relationships to disclose.

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inflammation Etymology: L, *inflammare*, to set afire; The protective or destructive response of body tissues to irritation or injury. Inflammation may be acute or chronic. Its cardinal signs are redness (rubor), heat (calor), swelling (tumor), and pain (dolor), often accompanied by loss of function. The process begins with a transitory vasoconstriction, and then is followed by a brief increase in vascular permeability. The second stage is prolonged and consists of sustained increase in vascular permeability, exudation of fluids from the vessels, clustering of leukocytes along the vessel walls, phagocytosis of microorganisms, deposition of fibrin in the vessel, disposal of the accumulated debris by macrophages, and finally migration of fibroblasts to the area and development of new, normal cells. The severity, timing, and local character of any particular inflammatory response depend on the cause, the area affected, and the condition of the host. Histamine, kinins, and various other substances mediate the inflammatory process. *inflammatory*, *adj.* Mosby's Medical Dictionary, 8th edition. © 2009, Elsevier.

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Abstract

Cancers often arise as the end stage of inflammation in adults, but not in children. As such there is a complex interplay between host immune cells during neoplastic development, with both an ability to promote cancer as well as limit or eliminate it, most often complicit with the host. In humans, defining inflammation and the presence of inflammatory cells within or surrounding the tumor is a critical aspect of modern pathology. Groups defining staging for neoplasms are strongly encouraged to assess and incorporate measures of the presence of apoptosis, autophagy, and necrosis as well as the nature and quality of the immune infiltrate. Both environmental as well as genetic factors enhance the risk of cigarette smoking, *H. pylori*, hepatitis B/C, human papilloma virus, solar irradiation, asbestos, pancreatitis, or other causes of chronic inflammation. Identifying suitable genetic polymorphisms in cytokines, cytokine receptors, and Toll-like receptors among other immune response genes is also seen as high value as genomic sequencing becomes less expensive. Animal models which incorporate and assess not only the genetic anlagen but also the inflammatory cells and the presence of microbial pathogen [PAMPs] and damage associated molecular pattern molecules [DAMPs] are necessary. Identifying micro-RNAs involved in regulating the response to damage or injury are seen as highly promising. Although no therapeutic strategies to prevent or treat cancers based on insights into inflammatory pathways are currently approved for the common epithelial malignancies, there remains substantial interest in agents targeting COX2 or PPAR γ , ethyl pyruvate, as well as steroids and several novel agents on the horizon.

Keywords

Chronic inflammation; damage associated molecular pattern molecules; pathogen associated molecular pattern molecules; miRNAs; cytokine polymorphisms; eosinophils; mast cells; NK cells; dendritic cells; COX2; ethyl pyruvate; steroids; HMGB1; TGF β

Introduction

How inflammation protects or destroys body tissues is indeed an important issue, particularly in the setting of cancer. The International Society for Biologic Therapy of Cancer [iSBTc] under the leadership of Dr. Bernard Fox, developed a one day workshop assessing “Cancer and Inflammation: Promise for Biologic Therapy” conducted in the Westin Gaslamp Quarter Hotel on October 30, 2008 in San Diego. In addition to the six sections: 1] *Defining Inflammation* cochaired by Michele Carbone, MD, PhD and Sandra Demaria, M.D.; 2] Genetic Polymorphisms and Factors which Modulate Inflammation and Cancer cochaired by Emad M. El-Omar, MB ChB, MD and Yen-Ching Chen, ScD, SM; 3] *Animal Models of Cancer and Inflammation* cochaired by Lisa M. Coussens, PhD and Michael Karin, PhD; 4] Causes and Molecular Targets in Cancer and Inflammation cochaired by Michael T. Lotze, MD and Giorgio Trinchieri, MD; 5] Current Clinical Evidence for Targeting Inflammation to Prevent

Cancer cochaired by Steven Dubinett, MD and Eva Szabo, MD; and 6] Novel Therapeutics and Clinical Trial Development to Treat Cancer cochaired by George J. Weiner, MD and Arthur M. Krieg, MD as state of the science sessions, breakout sessions reflecting these same areas were conducted and involved many individuals including Breakout Session 1: Robert Cardiff, Robert Edwards, Soldano Ferrone, Elliot Kagan, and Leif Hakansson; Breakout Session 2: Bernard Fox, Hazem Ghebeh, Jose Machado, Yeong-Shiau Pu, Senthamil Selvan, and Jianfeng Xu; Breakout Session 3: Robert Abraham, John Engelhardt, Alex Garcia, Daniel Hwang, Reginald Hill, Khashayarsha Khazaie, Eli Pikarsky, and Christian Poehlein; Breakout Session 4: Jason Gold, Craig Logsdon, Ainhua Perez-Diez, Steven Oh, Rimas Orentas, John Rediske, Michael Sheard, Geetha Srikrishna, and Antoine Tesniere; Breakout Session 5. Bharat Aggarwal, Harm-Jan Borgeld, Ezequiel Fuentes, Amy Fulton, Jenny Mao, and Augusto Ochoa; and Breakout Session 6: Sivasubramanian Baskar, Thomas Davis, Nathalie Dubois-Stringfellow, Jared Gollob, Toni Gray, John Kirkwood, Vladia Monsurro, Dolores Schendel, and Howard Streicher. The summaries below were derived from the chairs of the sessions and the participants noted above. A full review of the 2008 meeting and conclusions was given by Dr. Michael T. Lotze at the 2009 iSBTc Meeting held at the Gaylord National Hotel in National Harbor, MD on October 30, 2009.

Defining Inflammation

Inflammatory cells and soluble factors are present in all tumors. The signs of “smouldering” inflammation which include tissue remodeling, angiogenesis, and other wound healing-like features, are commonly used by pathologists as morphological cues of invasive cancer. Recent evidence demonstrates that these stromal processes play a fundamental role in cancer development and progression, and, at least in some cases, may predict the clinical behavior of a cancer better than the characteristics of the neoplastic cells themselves (1).

It has been known for some time that chronic inflammatory diseases increase the risk of cancer development in some organs (e.g., gastro-intestinal tract, prostate, thyroid gland, pancreas, urinary bladder, pleura, and others). For example, the chronic inflammatory response caused by asbestos has been linked to mesothelioma (2). However, the critical role of inflammatory cells in cancers that cannot be linked to a pre-existing inflammatory condition has been recognized only recently (3). Oncogenes target directly or indirectly pro-inflammatory pathways. For example, Ras activates the transcription of the inflammatory cytokine interleukin-8 (IL-8), whereas c-myc and bcl-2 inhibit apoptosis leading to necrotic tumor cell death and release of damage associated molecular pattern molecules or DAMPs (3,4). In both circumstances, the resulting host response is inflammation that promotes tumor invasion and growth (3,5).

Given what we are learning about the importance of the innate and adaptive immune system in tumor development, progression and metastasis, it is essential to revisit and update the diagnostic and prognostic criteria that have been traditionally employed to guide cancer staging and treatment. Whenever information about the predictive value of the inflammatory infiltrate is available from recently published studies, it should be incorporated into the pathological evaluation (“inflammation pathology”). Criteria for standardization, and requirements for validation need to be developed. We also need better markers for functional subtypes of leukocytes identified in pre-clinical studies as important players in order to address their role in human disease. Below we have summarized the current knowledge about inflammatory cells that have been demonstrated to significantly affect tumor pathogenicity.

Innate Immunity

The innate immune system functions as an “interpreter” of tissue damage that not only provides a first line of defense, but also translates the information to other repair and defense systems

in the body by stimulating angiogenesis, wound repair, and activating adaptive immunity. Therefore, it is not surprising that various types of innate immune cells have been found as part of the tumor inflammatory infiltrate. Among them, macrophages play a central role in most solid malignancies (2,6). Preclinical studies in rodent models of breast cancer have unequivocally demonstrated that macrophages promote invasion, angiogenesis and metastasis (7,8). The breast is peculiar in that macrophages are implicated in branching morphogenesis that occurs during puberty and pregnancy, and in post-weaning involution, suggesting that perhaps their role in breast cancer is an alteration of their physiological function (9). Macrophages are also present in all mouse and human tumors. Most studies have shown a correlation between their numbers, increased micro-vessel density, and reduced patients survival (6). In fact, macrophages present within tumors are defined as tumor-associated macrophages (TAM) to denote a specific phenotype that is associated with the production of many pro-angiogenic factors and immunosuppressive cytokines. However, TAM can also exhibit tumoricidal activity, and this could explain the reported association with improved prognosis in some cases (10,11). The location of TAM in hypoxic areas, stroma, or tumor cell nests may reflect their pro- or anti-tumor activity (6).

Immunosuppression of T cell-mediated anti-tumor responses has also been attributed to TAM and linked to their M2 polarization within the tumor microenvironment (12). However, myeloid cells expressing IL-4R α and derived from inflammatory type monocytes appear to be the key suppressors of activated anti-tumor CD8 T cells (13). A significant portion of IL-4R α myeloid-derived suppressor cells (MDSC) (14) acquire markers of mature macrophages in the tumor, suggesting that TAM and MDSC overlap. Their distinction within the tumor may be, to some extent, a matter of semantics (13).

Mast cells are commonly found in tumors. Pre-clinical data suggest that they contribute to tumor progression, perhaps by promoting angiogenesis (15,16). However, clinical data are contradictory showing association of mast cell numbers with poor survival in some studies, and with improved survival in others (11,17). Location in stroma or tumor cell nests, or degranulation of mast cells with release of heparin may be determinants of their pro- or anti-tumor effects (11,18).

Eosinophils are known to be associated with Hodgkin's lymphoma, but they are often present in solid cancers as well. Recent data suggest an important role for eosinophils in immunoregulation, however, their role in cancer remains unclear with reports of eosinophilia as good and bad prognostic factor (19). Eosinophilic granules contain a significant array of chemokines, cytokines and growth factors, suggesting that their ability to release some or all of these soluble mediators in individual tumor microenvironments may affect their pro- or anti-tumor effects (e.g., contribute to tissue repair or destruction) (19).

Dendritic cells are professional antigen-presenting cells that play an essential role in activation of adaptive immunity. Earlier studies have reported the association of DC infiltration of the primary tumor with significantly prolonged survival and reduced incidence of metastatic disease in patients with lung, stomach, and other cancers (20-23), but recent studies differentiating the maturation and subsets of DC indicate a more complex relationship between DC and tumors. For instance, the presence of CD123+ plasmacytoid DC (pDC) was associated with decreased survival in both breast and oral cancer (24,25). The significance of DC expressing the maturation markers CD208/DC-LAMP and/or CD83 remains controversial, since they have been reported not to be associated with clinical factors, to be associated with longer survival, or to be associated with lymph node involvement and tumor grade (24-26). The presence of mature DC at the edge of the tumor and their interaction with T cells may reflect an ongoing immune response (26,27). The nature of this response is difficult to establish in tissue sections, and could be a deleterious one promoting tumor growth (28).

Adaptive Immunity

The presence of tumor-infiltrating lymphocytes (TILs) has long been considered a manifestation of anti-tumor immunity, but until recently the prognostic significance of TILs was unclear. Development of markers that define the individual functional subsets of TILs has contributed to advances in this field (29). The presence of CD8+ T cells that express granzyme B (i.e., cytolytic T-cell, CTL) is a good prognostic factor in colorectal cancer, together with the location of TILs within tumor cell nests (30). A recent study employing gene expression profiling and immunohistochemistry (IHC) confirmed and extended these findings by demonstrating that the type, density and location of T cells in colorectal tumors is a better predictor of survival than the conventional histopathological criteria used to stage this cancer (31). The number of CD8+ TILs by itself has not correlated with survival in all malignancies. Preclinical studies have demonstrated that the ratio of effectors to regulatory TILs determines tumor rejection (32). Consistent with this notion, recent studies in patients with hepatocellular and ovarian carcinoma have shown that the ratio of CD8+/regulatory T cells (Treg, defined by FoxP3 expression) is an independent prognostic factor whereas the numbers of Treg and CD8+ TILs by themselves had lower or no predictive value, respectively (33,34). Since the discovery of FoxP3 as a marker of Treg there has been a proliferation of studies showing that FoxP3+ TILs correlate with reduced survival in many solid malignancies, whereas the opposite may be true for lymphoma (29). Overall, the large number of studies showing that the presence of CD8+ T cells together with markers of a Th-1 type effector response (1) predict a good prognosis, whereas the presence of Tregs is a predictor of worse prognosis, support the proposition that the most reliable prognostic indicators may be obtained by a comprehensive analysis of both CD8 (plus granzymes) and FoxP3 (29).

Although infiltration of tumors by B cells has not been shown to be significant, spontaneous humoral immune responses against tumors are so common that sera of cancer patients have been employed to identify many tumor-associated antigens (35). Rather than protective, humoral responses to cancer have been associated with a poor prognosis (36). In pre-clinical studies antibodies have been shown to promote cancer development by initiating local chronic inflammatory responses mediated by antibody and/or immunocomplex deposition, although in some cases the direct inhibition of Th1 responses by B cells may also play a role (37,38).

Important Questions And Issues

There are several issues which need further study. These are enumerated below:

- 1- Macrophages: how to differentiate TAM pro- and anti-tumor activities? Location within the tumor and/or markers? Most studies in humans use only CD68 as marker; what markers of functional differentiation (Legumain, Tie-2, IL-4R α) may be useful? Guidelines to define numerical categories of risk in a given tumor type are needed.
- 2- MDSC: validate the use of IL-4R α in human blood PBMC from cancer patients as a marker of immunosuppressive myeloid cells. Since the Workshop, the expression of IL-4R α has been shown to be a useful marker for MDSC identification in peripheral blood of cancer patients (39). How to evaluate the effects of pharmacological targeting of MDSC suppressive mechanisms in human tumors?
- 3- Mast cells: how to differentiate pro- and anti-tumor activities? Location within the tumor and/or markers of degranulation (e.g., tryptase, release of heparin)?
- 4- Eosinophils: how to define their role in cancer? Markers of function? Location?
- 5- Dendritic cells: given their functional heterogeneity, is it useful to analyze tumor-infiltrating DC? Are DC-LAMP and CD83 useful markers? Should we just stain tumors for IL-13 and/or pSTAT6?

6- TILs: should we always stain for CD8, granzymeB, and FoxP3 to obtain a more comprehensive and reliable prognostic indicator? Are we ready for prime time at least in colorectal, ovarian and hepatocellular cancers? Others? What is the role of Th17 in human cancer?

7- Sentinel lymph nodes (SLN): Should they be analyzed for the presence of immunological changes?

8- Methods for evaluation of prognostic/predictive parameters: Gene profiling assays for cancer (e.g., Oncotype DX, the 70-gene signature assay MammaPrint) are rapidly entering clinical practice. Should the “immunological signature” of a tumor be evaluated this way? Do morphology and IHC provide additional/different information? For instance, many studies emphasize the importance of location within the tumor (neoplastic cell nests versus stroma) of the effector T cells or the TAM, something that can be analyzed only by IHC. In addition, some markers (e.g., FoxP3) are expressed also by neoplastic epithelial cells so total tumor (not microdissected) should be used with caution for this analysis. On the other hand, there are some known limitation in IHC analysis, as apparent from biomarkers routinely evaluated by IHC (e.g., Hormone receptors, HER-2 in breast cancer) that suffer from variability in staining and interpretation.

9 Defining an Immunologic Signature: Besides prognostic value in terms of the natural behavior of the cancer, the “immunological signature” of a tumor may be an important predictor of response to immunotherapy. Current trials of immunotherapy do not tailor treatment to the patient/tumor type, possibly a major factor in the observed clinical response.

Genetic Polymorphisms and Factors which Modulate Inflammation and Cancer

The completion of the human genome project was a momentous occasion for humanity. It opened up the opportunity to dissect complex human traits and to understand basic pathways of health and disease. Population-based association studies have emerged as powerful tools for examining genes with a role in common multifactorial diseases that have a strong environmental component. These association studies often estimate the risk of developing a certain disease in carriers and non-carriers of a particular genetic polymorphism. The overwhelming majority of polymorphisms studied are single nucleotide polymorphisms (SNPs) that occur with a frequency of > 1% in the normal population (in contrast to “mutations” that occur with a frequency of < 1%). It is estimated that up to 10 million SNPs are probably present in the human genome though not all have thus far been identified. Naturally, most of these SNPs do not occur in coding sequences and even those that do, are not associated with any alteration in the amino acid sequence and are therefore of no functional consequence. There has been an exponential rise in the number of published genetic association studies. Quite often, a report of a single genetic marker is published with great promise only to be followed by several negative studies that fail to reproduce the original observation. There is no doubt that the strategy of genetic association studies could be a powerful tool for dissecting human diseases, provided certain principles are observed to minimize the chances of false positive, and negative, reports. In the following sections, we will discuss the role of genetic polymorphisms that modulate inflammation and risk of cancer. We will use two specific cancer models, prostate and gastric cancer, to demonstrate the principles involved.

Prostate Cancer

In the USA, prostate cancer has been the most common non-skin cancer in men and the 2nd most common cause of cancer-related death. In a study on twins, 42% of prostate cancer cases up to age 70 years could be explained by heritable factors. The proportions were lower for

colorectal cancer (35%) and breast cancer (27%) (40). This highlights the important role of genetic factors in the pathogenesis of this cancer. Some studies observed that Asians who immigrated to the West later in their life had lower risk of prostate cancer as compared to Caucasians (41,42). In addition, a recent study found that *Propionibacterium acnes* was detected in 35% of radical prostatectomy specimens (43). These implied that not only genetic factors but also environmental factors (e.g., lifestyle or microbial infection and underlying subclinical prostatitis) might play a role in prostate carcinogenesis. Three genetic epidemiologic approaches: candidate gene approach, pathway analysis, and genome-wide association studies, have been used to assess genetic polymorphisms and the risk of prostate cancer.

Candidate Gene Approach

The candidate gene approach is a hypothesis-driven method that has been widely employed. Sequence variants of several inflammatory genes (e.g., RNASEL, MSR1, TLRs, MIC1, TNF- α , TNF-R β 1, IL1B, IL6, IL8, IL10, IL1RN, VEGF, and COX2, etc.) have been extensively explored to predict prostate cancer risk (44,45). However, the findings are inconsistent. Studies on genetic polymorphisms of *Toll-like receptor 4 (TLR4)* are used to demonstrate the inconsistent findings across studies by using the candidate gene approach. To date, three studies have been performed to assess genetic polymorphisms of *TLR4* and the risk of prostate cancer. Chen et al (46) reported significant association between 10 SNPs, 1 haplotype and decreased risk of prostate cancer, in which fewer high-grade prostate cancer cases were included. In contrast, Zheng et al (47) included more high-grade prostate cancer cases (17%) compared to Chen et al. (8%) and found one SNP associated with increased risk of prostate cancer. The other study (48) only recruited advanced prostate cancer and they observed two SNPs significantly associated with increased risk of prostate cancer. These inconsistent findings may result from distinct populations, study design, selection of SNPs, and characteristics of the cases analyzed (e.g., advanced or high-grade cases), etc.

Pathway Analysis

The pathway analysis approach, which is also hypothesis driven, relies on examining a more comprehensive set of genes involved in a specific functional role, e.g. inflammation, cell cycle, DNA repair etc. However, this approach is being overtaken by advances in high-throughput genome technology and the advent of genome-wide association studies. A case-control study (49) used the multiple-stage design to assess the association between genetic polymorphisms of over a thousand inflammatory genes and the risk of prostate cancer. Three SNPs, rs7250623 on *CRLF1* gene, rs753733 on *FCER2* gene, and rs2144493 on *CIDEB* and *LTB4R2* genes, are associated with the risk of prostate cancer (49). However, these SNPs are not the most significant SNPs and represent a small fraction of true associated SNPs; therefore, additional SNPs with lower significance level may contain true associated SNPs (49). Even though this approach allows us to explore the sub-pathway interactions, it limits the ability to assess the cross talk between the inflammation pathway and other pathways.

Genome-Wide Association Study

In the post genome era, genome-wide association study (GWAS) has become a powerful tool to screen the whole genome and identify SNPs related to the outcome of interest. Several GWAS have been done for prostate cancer and the results have been quite consistent (50-52). An excellent example that demonstrates recent advances in GWAS is the study by Zheng et al (53). They selected 16 SNPs that were significantly associated with the risk of prostate cancer in previous GWAS. These SNPs are located at 5 chromosomal regions and the most significant SNP can be selected from each region. Among the top significant 5 SNPs, only one SNP located at *TCF2* gene and the others are located at non-coding region. The population attributable risk

of 5 SNPs plus family history is 46%, which could be a very useful tool in predicting the risk of prostate cancer in the future. The less SNP identified associated with genes encoding pro-inflammatory proteins might be due to multiple variants such as different technologies used, improper comparison analysis algorithm, sample size limitation and technology platform coverage range. Therefore, higher false positive rates are observed for GWAS. As the array based SNP detection resolution and the throughput across genome increase dramatically, the accuracy increments will reverse correlate with the faults discovery rate. The application of next generation of sequencing technology in genetic association study will bring genetic study into a new era and accelerate SNP discovery and improve the accuracy of SNP association with physiological or pathological conditions. Genome wide de novo sequencing of large number of samples in a given condition will also provide the opportunity to discover new monogenic and polygenic association with phenotype or outcome.

Gastric Cancer

Globally, gastric cancer is the second most common cause of cancer-related death and, as a result of population aging and growth, the predicted incidence for 2010 is 1.1 million with the majority of this health burden being borne by economically lesser-developed countries (54). Here, we hope to shed some light on the role of host genetic susceptibility in the pathogenesis of gastric cancer. In particular, we will demonstrate how interactions between an infectious agent that causes chronic inflammation, host genetic makeup, and environmental factors could influence the pathogenesis of this cancer. The infectious agent in question is *Helicobacter pylori* (*H. pylori*), the world's commonest chronic bacterial infection.

Role of Interleukin-1 (*IL-1*) Genetic Markers in Gastric Cancer

H. pylori causes its damage by initiating chronic inflammation in the gastric mucosa. This inflammation is mediated by an array of pro- and anti-inflammatory cytokines. Genetic polymorphisms directly influence inter-individual variation in the magnitude of cytokine response and this clearly contributes to an individual's ultimate clinical outcome. In the case of *H. pylori* infection, the most relevant candidate genes are ones whose products are involved in handling the *H. pylori* attack (innate and adaptive immune responses) and ones that mediate the resulting inflammation. Because such a list of candidate genes would be prohibitively extensive, the initial search focused on genes that were most relevant to gastric physiology, and in particular, gastric acid secretion. *H. pylori*-induced gastritis is associated with three primary phenotypes that correlate closely with clinical outcome: duodenal ulcer (DU) phenotype, benign phenotype and gastric cancer phenotype (55). Inhibition of gastric acid pharmacologically can lead to a shift from an antrum-predominant pattern (DU phenotype) to a corpus-predominant one with onset of gastric atrophy (gastric cancer phenotype) (56). Thus it is clear that an endogenous agent is upregulated in the presence of *H. pylori*, and has a profound pro-inflammatory effect. An acid inhibitor could be the most relevant host genetic factor to be studied. Interleukin 1 beta (*IL-1* β) fit this profile perfectly, for not only is it one of the earliest and most important pro-inflammatory cytokines, in the context of *H. pylori* infection, it is also the most powerful acid inhibitor (57). Pro-inflammatory *IL-1* gene cluster polymorphisms (*IL-1B* encoding *IL-1* β and *IL-1RN* encoding its naturally occurring receptor antagonist) increase the risk of gastric cancer and its precursors in the presence of *H. pylori*. Individuals with the *IL-1B*-31*C or -511*T and *IL-1RN**2/*2 genotypes are at increased risk of developing hypochlorhydria and gastric atrophy in response to *H. pylori* infection. This risk also extends to gastric cancer itself with a 2-3 fold increased risk of malignancy compared to subjects who have the less pro-inflammatory genotypes (58,59).

Furthermore, the pro-inflammatory *IL-1* genotypes increase the risk of both intestinal and diffuse types of gastric cancer but the risk is restricted to the non-cardia subsite. Indeed, *IL-1*

markers have no effect on risk of cardia gastric adenocarcinoma, esophageal adenocarcinoma or esophageal squamous cell carcinoma (59). The association between *IL-1* markers and gastric cancer in Caucasians has been confirmed with similar odds ratios reported (60). The combined effects of pro-inflammatory *IL-1* genotypes and *H. pylori* bacterial virulence factors (*cagA* positive, *VacA s1* and *VacA m1*) seem critical. For each combination of bacterial/host genotype, the odds of having gastric carcinoma are greatest in those with both bacterial and host high-risk genotypes (61). This highlights the important interaction between host and bacterium in the pathogenesis of gastric cancer.

A crucial piece of evidence that confirmed the unique role of IL-1 β in *H. pylori*-induced gastric carcinogenesis came from a transgenic mouse model in which IL-1 β overproduction was targeted to the stomach by the H+/K+ ATPase beta promoter. With overexpression of IL-1 β confined to the stomach, these transgenic mice had a thickened gastric mucosa, produced lower amounts of gastric acid and developed severe gastritis followed by atrophy, intestinal metaplasia, dysplasia and adenocarcinoma. Crucially, these IL-1 β transgenic mice proceeded through a multistage process that mimicked human gastric neoplasia. These changes occurred even in the absence of *H. pylori* infection, which when introduced led to an acceleration of these abnormalities (62).

Role Of Other Cytokine Gene Polymorphisms In Gastric Cancer

Soon after the *IL-1* gene cluster polymorphisms were identified as risk factors for gastric cancer, the pro-inflammatory genotypes of tumor necrosis factor- α (*TNF-A*) and *IL-10* were reported as independent additional risk factors for non-cardia gastric cancer (59). TNF- α is another powerful pro-inflammatory cytokine that is produced in the gastric mucosa in response to *H. pylori* infection. Like IL-1 β , it has an acid inhibitory effect, albeit much weaker (63). The *TNF-A-308 G>A* polymorphism is known to be involved in a number of inflammatory conditions. Carriage of the pro-inflammatory A allele increased the odds ratio for non-cardia gastric cancer to 2.2 (95% confidence intervals: 1.4-3.7). The role of the *TNF-A-308 G>A* polymorphism in gastric cancer was independently confirmed by a study from Machado et al (64). IL-10 is an anti-inflammatory cytokine that downregulates IL-1 β , TNF- α , interferon- γ and other pro-inflammatory cytokines. Relative deficiency of IL-10 may result in a T helper-1 (Th-1) -driven hyper-inflammatory response to *H. pylori* with greater damage to the gastric mucosa. Homozygosity for the low-IL-10 ATA haplotype (based on three promoter polymorphisms at positions -592, -819 and -1082) increase the risk of non-cardia gastric cancer with an odds ratio of 2.5 (95% CI: 1.1-5.7).

Having more pro-inflammatory genotypes (*IL-1B-511*T*, *IL-1RN*2*2*, *TNF-A-308*A*, and *IL-10 ATA/ATA*) enhances the risk of non-gastric cancer. The risk increases progressively so that by the time 3-4 of these polymorphisms are present, the odds ratio for gastric cancer increases 27-fold (59). The fact that *H. pylori* is a pre-requisite for the association of these polymorphisms with malignancy demonstrates that in this situation, inflammation is indeed driving carcinogenesis.

Role Of Polymorphisms In The Innate Immune Response Genes

Genetic polymorphisms of cytokines of the adaptive immune response clearly play an important role in the risk of *H. pylori*-induced gastric adenocarcinoma. However, *H. pylori* is initially handled by the innate immune response and it is conceivable that functionally relevant polymorphisms in genes of this arm of the immune system could affect the magnitude and subsequent direction of the host's response against the infection. *H. pylori* does not typically invade the gastric mucosa but the inflammatory response against it is triggered through attachment of *H. pylori* to the gastric epithelia (65). TLR4, the lipopolysaccharide (LPS) receptor, was initially identified as the potential signalling receptor for *H. pylori* on gastric

epithelial cells (66). TLR4 belongs to a family of pattern recognition receptors that activate pro-inflammatory signalling pathways in response to microbes or pathogen-associated molecular patterns (PAMPs) (67). TLR4, in conjunction with CD14 and MD-2, transduces signals through MyD88, Toll/IL-1 receptor domain and TRAF6. This promotes transcription of genes, which are involved in immune activation including the transcription factor NF- κ B and also MAP kinase pathways (68).

A functional polymorphism at position +896 in exon 4 of the *TLR4* gene (dbSNP ID: rs4986790) (69) has been reported. This A>G transition results in replacement of a conserved aspartic acid residue with glycine at amino acid 299 (Asp299Gly), and alteration in the extracellular domain of TLR4. This renders carriers hyporesponsive to LPS challenge by either disrupting transport of TLR4 to the cell membrane or by impairing ligand binding or protein interactions (69). Recent work demonstrates that defective signaling through TLR4 ultimately leads to an exaggerated inflammatory response with severe tissue destruction, even though the initial immune response may be blunted. This is due to inadequate production of IL-10-secreting type 1 regulatory cells (70). *H. pylori* *TLR4*+896G carriers have a 7.7-fold (95% CI, 1.6-37.6) increased odds ratio for hypochlorhydria. The polymorphism is not however associated with gastric acid output in the absence of *H. pylori* infection. Carriers also have significantly more severe gastric atrophy and inflammation (71). The polymorphism also increased the risk of non cardia gastric cancer (OR=2.4; 95% CI=1.6-3.4) (71).

The association of *TLR4*+896A>G polymorphism with both gastric cancer and its precursor lesions implies that it is relevant to the entire multistage process of gastric carcinogenesis, which starts with *H. pylori* colonization of the gastric mucosa. Subjects with this polymorphism have an increased risk of severe inflammation and subsequently, development of hypochlorhydria and gastric atrophy, which are regarded as the most important precancerous abnormalities. Severe inflammation is initiated by *H. pylori* infection but it is entirely feasible that subsequent co-colonization of an achlorhydric stomach by a variety of other bacteria may sustain and enhance the microbial inflammatory stimulus and continue to drive the carcinogenic process.

Thus, it appears that subjects with a pro-inflammatory genetic makeup based on a combination of markers from the adaptive immune response (e.g. IL-1 β , TNF- α , IL-10, IL-8) and the innate immune response (e.g. TLR4), respond to *H. pylori* infection by creating an environment within the stomach that is chronically inflamed and with reduced acidity. This environment is conducive to the growth of other bacteria within the gastric milieu, leading to sustained inflammation and oxidative/genotoxic stress. Subjects with the same pro-inflammatory polymorphisms may respond in the same exaggerated manner to these non-*H. pylori* bacteria, thus maintaining the pro-neoplastic drive. This may explain why *H. pylori* is not required in the latter stages of gastric carcinogenesis and why it is often absent from gastric tumour tissue.

Important Questions And Issues

There are several areas worthy of further investigation.

1-Gene and Environment Issues: It is important to explore these interactions in order to fill the gap of current studies that focus on cancer treatment. Studies including different ethnic groups could also help us to elucidate the role of gene and environment as well as their interactions in carcinogenesis. Genome-wide approach not only allows us to evaluate the association within one pathway, it also provides the possibility to explore the interactions between different biological pathways. Importantly, data from multiple levels (DNA, RNA, or protein levels) allowing us to confirm the association between the identified markers and the outcome of interest, e.g., cancer prognosis after treatment, cancer risk, or cancer mortality.

2-Intensively Study Gastric Cancer: Sporadic gastric cancer is a common cancer with a grave prognosis, particularly in the West. A major advance came with the recognition of the role of *H. pylori* infection in its pathogenesis. The cancer represents a classic example of an inflammation-induced malignancy. Host genetic factors, interacting with bacterial virulence and environmental factors, play an important role in the pathogenesis of this cancer. In particular, genetic polymorphisms in the adaptive and innate immune response genes seem to increase the risk of cancer, largely through induction of severe gastritis, which progresses to atrophy and hypochlorhydria. The pro-inflammatory host genetic makeup is only relevant in the presence of infection, initially *H. pylori* but later other bacteria that thrive in an achlorhydric environment. Future research must focus on defining a more comprehensive genetic profile that better predicts the clinical outcome of *H. pylori* infection, including gastric cancer and finding cost-effective means to eradicate the bacteria. Genetic profiling in combination with testing for the infection and its virulence factors may prove a useful tool in targeting the populations that require eradication therapy. Eradication studies aiming to prevent non-cardia gastric cancer should also focus on identifying who might develop an unfavorable outcome to this strategy. Host genetics will no doubt play its role in defining these patients as well.

3-Intensively Study Prostate Cancer: This tumor type has profound genetic relationship based on twin studies. It should be possible to define the genetic anlagen of this high incidence tumor type in more detailed studies.

Studying Inflammation And Tumor Cross-Regulation And Its Impact On Cancer Progression In Mice

Examination of genetically modified or chemically-induced mouse models susceptible to *de novo* tumor development, where selective components of the immune system have been deleted or modified, have provided important clues regarding the functional significance of specific immune response programs as regulators of tumor immunity. Since chronic inflammation is a complex and dynamic process involving multiple cell types and soluble mediators, it is not surprising that diverse mechanisms have been identified whereby inflammation promotes malignancy (72,73). Developing neoplasms contain diverse leukocyte populations, including neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes. Moreover, it is now realized that each of these cell types can adopt various phenotypes and bioeffector programs, which can coexist in the same tumor and differentially affect tumor progression via many different mechanisms (2).

Cancer And Inflammation In Murine Models

It is clear that one of the manifestations of the inflammatory microenvironment is suppression of anti-tumor immune responses. Thus, the effect of inflammation on anti-tumor immunity needs to be considered if we want to fully understand how chronic inflammation promotes tumor development. Inflammation is not one response but instead represents a dynamic and continuously changing micro-environmental process that has various effects at subsequent stages of tumorigenesis. An important aspect that still needs to be better defined is co-evolution of the tumor as it relates to the inflammatory process and the degree to which this represents a tissue microenvironment-specific process. While defining the various aspects of this co-evolution can and should be done in human tissues, it is important to identify mouse models that recapitulate the human changes in order to dissect the functional role of each specific change. Primary tumor mouse models with predictable disease progression, akin to the human counterpart should be used for this purpose.

Several experimental pitfalls and tips should be underscored: 1) Only immune competent mice should be used to study the role of inflammation in cancer. Moreover, xenografts nearly always

produce an acute immune response and we therefore discourage their use in this setting. 2) Strain differences modify tumor phenotypes and tumor penetrance as well as the immunophenotype accompanying tumor development. For example, C57Bl/6 mice are biased towards a T_H1 response while FVB mice are biased instead towards a T_H2 response. 3) Presence of pathogens in an animal facility also dramatically alters experimental results. Two important pitfalls are perhaps best exemplified by the erroneous conclusion by Dr. Johannes Fibiger that Nematodes are the cause of stomach cancer that led to his award of the Nobel prize in medicine (74). The first pitfall is overlooking the possibility that a specific intervention may be inducing additional changes other than those expected, in this case vitamin A deficiency. 4) Another pitfall to avoid is interpretation of histopathological data. It is highly recommended that an experienced pathologist be involved from the planning stage in experiments involving animal models of cancer.

There are two main methodological approaches that can be used in mouse models, a cell based approach – targeting distinct immune cell lineages, and a signaling based approach - targeting specific signaling pathways either in epithelial cells or in immune cells. An emerging theme is that *context matters*, i.e. – different tumor types and models will reveal different roles for individual cell types. Similarly, etiology is important. Thus, defining the whole picture requires meticulous analysis of multiple tumor models, the choice of which may dramatically affect the progress of knowledge. The best guiding principle for model choice should be human relevance (Table 1).

Important questions and issues

There are several areas worthy of further investigation.

- 1- Identify Cells and Molecular Components: Can we identify cellular and molecular components that are common to all cancer-promoting inflammatory responses?
- 2- Identify Bioactive Mediators: Innate immune cells directly and indirectly potentiate cancer risk through the diversity of bioactive mediators they deliver to neoplastic tissues. While the evidence for some mediators is strong (MMPs, some cytokines, angiogenesis), for others there is less evidence (reactive oxygen species, reactive nitrogen species). Targeting them pharmacologically may be important.
- 3- Innate Immune Cells in Murine Models: Define the phenotypes and subtypes of hematopoietic cells (leukocytes, monocytes, mast cells, platelets, etc) involved in tumor initiation and progression and characterize their role. Define the physiological roles of the pro-tumorigenic subtypes of immune cells and study the possible side effects [immunodeficiency] resulting from neutralizing the pro-tumor properties of these cells utilizing immuno-depletion or pharmacologic inhibition strategies.
- 4- Timing and Location: We need to better define the role of the various immune cells in the individual stages of tumorigenesis, beginning in the cancer prone chronically inflamed tissue, through cancer initiation, promotion, progression and metastasis.
- 5- Pharmacologic Strategies: How does long-term usage of non-steroidal anti-inflammatory drugs reduce cancer recurrence, and determine if COX-2, or perhaps other proteins involved in prostaglandin biosynthesis represent the best targets?
- 6- The Adaptive Immune System: Whereas some studies have provided convincing data supporting the concept that the immune system exerts a protective role against certain tumor types, other studies show enhanced tumor progression in some settings; thus malignant outcome is etiology-, context- and organ-dependent.

Proximal Factors in Cancer: DAMPs and PAMPs

Field effects in cancer have been described for over fifty years. Histologically abnormal tissues surround carcinomas of the head and neck, bladder, prostate, lung, esophagus, vulva, cervix and breast. Although not frankly cancer, these changes in cellular architecture and the normally ordered progression of adjacent epithelia are disturbed. Field effects have been associated with genetic alterations due to postulated 'field carcinogenesis' driven by common mutagens, alterations in DNA methylation, and other epigenetic changes (75). Recent studies have revealed that persistent pro-tumor immune responses (inflammation), now generally believed to be important in primary tumor development, can also potentiate and lay the seeds for cancer metastasis and may represent a component of the field effect. Tumor metastasis into the liver triggers a profound inflammatory cascade that begins with the release of TNF- α and IL-1 β by activated Kupffer cells and leads to rapid expression of E-selectin and other vascular adhesion receptors such as ICAM-1 and VCAM-1 on the hepatic sinusoidal endothelial cells. These initial events facilitate tumor cell transmigration from vessels into the extra-vascular space. Stressed tissues limit cellular apoptosis and promote programmed cell survival and enhanced autophagic flux. This is associated with release of DAMPs that include Interleukin 1 α , HMGB1 and other reducing cellular contents of cells that directly promote these tissue architectural changes. In the setting of chronic tissue injury and cancer, we hypothesize that the normal tissue architecture is perturbed and associated not only with the release of HMGB1 but also with the activation of latent TGF β . The balance between HMGB1 and TGF β are particularly critical for altering the recruitment and activation of inflammatory cells, promoting a disturbed redox environment, breakdown in normal orderly tissue maturation, and driving the field effects associated with autophagic release of HMGB1.

We hypothesize that cancer fundamentally, is a disorder of cellular and tissue architecture driven by redox and DAMPs. Stressed cells release into the tumor microenvironment DAMPs which interact with their cognate receptors (DAMP-R) such as the Receptor for Advanced Glycation Endproducts [RAGE] on surviving, stressed cells within the tumor microenvironment, where they drive a disordered tumor microenvironment. This disordered microenvironment favors tumor cell resistance to therapy by limiting apoptosis, enhanced stromagenesis, angiogenesis, and suppression of the adaptive immune response.

Damage-associated molecular pattern (DAMP) molecules are nature's alarm signals, initiating and propagating host immune responses against insults or events (e.g. infections, tumor metastasis). High mobility group B1 molecule (HMGB1) (76) is a DAMP passively released from necrotic tumor cells or actively secreted by macrophages or hepatocytes into the local microenvironment. As a nuclear DNA-binding protein, HMGB1 plays a role in the transcription of several genes, some of which include those that have been implicated in cancer development such as E-selectin, TNF- α , insulin receptor, and BRCA. Extracellular HMGB1 can lead to acute responses to ischemia/reperfusion (77-79) and chronic inflammatory/ reparative responses that, in the setting of cancer, may lead to tumor cell survival, expansion, and metastases. As a proinflammatory cytokine, HMGB1 may signal through the receptor for advanced glycation end products (RAGE) or via TLR2, TLR4, and/or potentially other TLRs in association with other ligands. Through TLRs HMGB1 activates NF κ B inducing a wide range of host changes that include 1) activation of the innate immune system (neutrophils, NK cells, dendritic cells) (80-82) and secretion of pro-inflammatory cytokines and mediators; 2) activation of endothelial cells and angiogenesis; and 3) stem cell migration and cell motility and proliferation. HMGB1 plays a role in metastasis development and thus links it to poor prognosis in a variety of cancers including prostate, breast, pancreas, and colon. The sum of these findings strongly suggests that HMGB1 plays a role in tumor development, growth, and metastasis and, thus warrants further investigation as a possible therapeutic target. One of the central hypotheses is that HMGB1 as a DAMP released into the tumor microenvironment plays

a central role in the growth of tumors by its recruitment and activation of innate immune cells, with the resulting chronic inflammatory milieu promoting stromagenesis, angiogenesis, and cell proliferation, thus enhancing tumor growth.

The Fate of Tumor Cells: Survival/Autophagy, Apoptosis, or Necrosis

Necrosis is morphologically characterized by swelling of the cytoplasm and oncosis, leading to the rupture of the plasma membrane, and the release of swollen and damaged organelles. Necrosis is usually considered to be immunologically harmful because of the sudden release of proinflammatory mediators. Necrotic cell death causes the release of proinflammatory cytokines such as interleukin-8 (IL-8), IL-10, TNF- α , or of terminal mediators of inflammation, such as HMGB1. The release of HMGB1, TNF- α , etc., promotes a chronic inflammatory process that may favor tumor growth. Thus, agents that cause cell necrosis, rather than apoptosis, may be carcinogenic.

Apoptosis is primarily defined by its morphological hallmarks, including chromatin condensation, nuclear fragmentation, shrinkage of the cytoplasm, and formation of apoptotic bodies. Apoptosis in cancer cells can be induced by hypoxia, shortage of nutrients or growth factors, and radiotherapy or chemotherapy. As a means of protecting the host, physiological apoptosis is rapidly and specifically recognized by phagocytic cells. Apoptotic bodies are silently removed by phagocytosis; this event is associated with the release of potent anti-inflammatory mediators like transforming growth factor- β (TGF- β), prostaglandin E2, or platelet-activating factor in order to avoid local inflammatory reactions. Therefore, apoptosis has been unanimously considered as an immunologically silent type of cell death. Apoptosis eliminates cells that have accumulated DNA damage without causing inflammation. Thus apoptosis prevents tumor formation first and tumor growth later. Apoptosis is a mechanism of self-destruction that involves mitochondria; notably, this mechanism fails in cancer cells with expression of anti-apoptotic proteins. A major goal of cancer therapy is reactivation of an apoptotic program.

Autophagy, literally self-eating, is an important mechanism in which eukaryotic cells respond to cellular stress and provide routine housekeeping functions to remove long lived proteins and dysfunctional organelles. In response to environmental stress, autophagy provides the bioenergetic needs of the cell necessary to program cell survival and adapt to stress through catabolic activity. If a cell is stressed for a prolonged period, autophagy may induce cell death, although this is a rather unusual event and it is most appropriate to consider autophagy a means for “programmed cell survival” balancing and counter-regulating apoptosis. Autophagy appears to have a dichotomous role in tumorigenesis and tumor progression. When baseline levels of autophagy are compared in many cancer cells and non-cancerous cells from the same tissue, decreased autophagy is observed in many cancer cells. Inhibiting autophagy can promote carcinogenesis by encouraging increased levels of protein synthesis and decreased levels of degradation, increasing unrepaired and accumulated mutations, and removing the suppressive effects of oncogenes associated with increases in damaged organelles, producing additional genotoxic stress such as generation of reactive oxygen species and free radicals (83-85). In response to hypoxia, acidosis, or nutrient deprivation, autophagic flux is accelerated in cancer cells in the later stages of tumor progression. As the tumor enlarges the cells adjacent to blood vessels with proper nutrients and oxygen supply favor anabolism.

The cells within the center of the tumor, deprived of an adequate blood supply have upregulated autophagic flux to allow for survival in the hypoxic and low nutrient microenvironment (86-88). Increased autophagic flux is observed in late stage colon cancer, breast cancer, melanoma, hepatoma, and malignant glioma. Many cancer therapies considered over the last couple of years have been thus paradoxically aimed at either inducing or reducing levels of

autophagy. Therefore, it is essential to understand the role of autophagy in different stages of cancer development and progression, and identify the autophagic pathways in cancer cells and how they can be modified to enhance response to therapy.

HMGB1 (89-95) translocated to the cytosol in the setting of autophagy can bind Beclin-1 with dissociation of Beclin-1/Bcl-2 [Tang DL, Kang R, Zeh H, Lotze MT et al, submitted]. Mitochondrial HMGB1 regulates cellular bioenergetics and mitophagy by promoting phosphorylation and activation of ERK1/2 (pERK1/2). Reduced but not oxidized HMGB1 suppresses SOD and mTOR expression, and increases mitochondrial superoxide production, which in turn induces autophagy. This promotes recruitment of inflammatory cells including macrophages and results in the profound cascade of cytokines and chemokines that has been found in the serum of patients (96). Oxidative denaturation of the DAMPs allows resolution (97,91,93). The critical interface between tolerance and immunity is dictated by oxidation or reduction of HMGB1 (98). When first released HMGB1 is reduced and promotes immunity and with resolution of inflammation, it is oxidized and inactivated [and we postulate in turn TGF β is activated].

TGF- β Family

The peptide structures of the three members of the TGF- β family are all encoded as protein precursors. TGF- β 1 [390 amino acids] and TGF- β 2/TGF- β 3 [412 amino acids]. They encode an N-terminal signal peptide of 20-30 amino acids that is required for secretion, a pro-region (latency associated peptide, LAP), and a 112-114 amino acid C-terminal region that becomes the mature TGF- β molecule following its release from the pro-region following proteolytic cleavage (99-102). TGF- β dimerizes to produce a 25 KDa active molecule with nine cysteine residues, conserved among its family. Eight disulfide bonds form within the molecule to create a cysteine knot structure characteristic of the TGF- β superfamily. The ninth cysteine forms a bond with the ninth cysteine of another TGF- β molecule to produce the dimer. Other conserved residues in TGF- β form secondary structure through hydrophobic interactions. The region between the fifth and sixth cysteines is the most divergent area of TGF- β . It is exposed at the surface of the molecule following oxidative activation (101,102) and is implicated in receptor binding and specificity of TGF- β .

Autophagy Serves As A Survival Pathway During Genotoxic And Metabolic Stress

Autophagy in mammalian cells is under the control of the mammalian target of rapamycin (mTOR), which suppresses autophagy and enhances transcriptional activity in response to nutrient availability (94). Phosphorylation of mTOR makes it a more potent inhibitor of autophagy. Release of suppression allows formation of a multiprotein complex that includes class III phosphatidylinositol 3-kinase (PI3K), Beclin 1, and vacuolar protein sorting factor protein 15 (Vps15). In both cancer as well as normal cells with defects in apoptosis, autophagy allows prolonged survival. In landmark experiments suppression of apoptosis and autophagy in immortalized but otherwise nontransformed renal epithelial cells leads to increased necrotic cell death, genomic instability, inflammation and rapid development of cancer. We would explain these results by positing that prevention of autophagy accelerates tumor promotion by enhancing necrotic tumor cell death and consequent release of DAMPS (94,95). Promotion of autophagy as a therapeutic strategy has been based on the concept of autophagic cell death. Although autophagy was initially described as a non-apoptotic pathway of programmed cell death, it now appears that in most circumstances it primarily serves as a survival mechanism by which stressed or dying cells limit apoptosis and necrosis (94). Inhibition of autophagy enhances traditional cytotoxic tumor therapies. HMGB1 leads to inflammation and increased tissue damage in a murine model of ischemia reperfusion, signaling through TLR4 (89,90,

93). In this model blockade of extra-cellular HMGB1 with neutralizing antibodies or disabling of the TLR4 receptor leads to decreased local expression of inflammatory cytokines (IL-6, TNF), decreased histologic evidence of inflammation and reperfusion injury and alteration in inflammatory intracellular signaling pathways. TLR4 expression appears to be most important on myeloid cells as bone marrow chimeras made from TLR deficient mice are protected from ischemia reperfusion injury similar to blockade of HMGB1. HMGB1 is released into the systemic circulation following warm ischemia of the liver. Systemic release of HMGB1 was associated with increases in serum IL-6 and TNF- α . In this model blockade of HMGB1 led to a decrease in the overall inflammatory response and better overall survival. Administration of soluble RAGE, one of the receptors for HMGB1, attenuates reperfusion/ischemia injury. Together these studies support a role for DAMPs and their receptors in non-infectious models of tissue damage and inflammation including cancer.

Ethyl Pyruvate: Example of an Anti-Inflammatory Agent With Novel Anti-Tumor Effects

Pyruvate, a key metabolite in cellular energy metabolism, is the end-product of glycolysis and the starting substrate for the tricarboxylic acid cycle which generates NADH required for ATP synthesis in oxidative phosphorylation. Pyruvate displays anti-inflammatory as well as anti-oxidant properties, ameliorating ischemia-reperfusion injury in a variety of animal models. Other studies, however, have demonstrated a lack of such activity. EP, the ethyl ester of pyruvate, improves survival and organ dysfunction in animal models of severe sepsis, ischemia-reperfusion, acute pancreatitis, and stroke (103-105). *In vitro* studies have suggested that the ethyl moiety as well as delivery of the intact EP molecule is required for the anti-inflammatory effects of EP, as the combination of ethanol and pyruvate did not suppress the inflammatory response of endothelial cells. EP improves survival and organ dysfunction in both large and small animal models of endotoxemia, sepsis, ischemia-reperfusion, acute pancreatitis, etc, by exerting potent anti-inflammatory effects through the inhibition of the production and release of cytokines (TNF, IL-1, IL-6) and other pro-inflammatory mediators such as HMGB1. The precise mechanisms by which EP exerts its anti-inflammatory effects have not been completely elucidated, but there are likely multiple pathways. Numerous studies have shown that EP decreases HMGB1 release, and other studies have shown that it strongly inhibits NF κ B activation. EP also ameliorates hepatic ischemia-reperfusion by decreasing hepatocyte apoptosis. Methyl-2 acetamidoacrylate (M-2AA) is an EP analog that is 100-fold more potent than EP in inhibiting TNF and nitric oxide production (103). M2AA administration at the time of cecal ligation and puncture in mice improves survival, renal function, liver injury, and lowered pro-inflammatory cytokine levels. Importantly, EP has been tested in Phase II trials of high risk cardiac patients undergoing cardiopulmonary bypass and has been shown to be safe and well-tolerated. Although there was no benefit conferred to these patients, EP has never been tested in the cancer setting in humans.

Lipid DAMPs

Inflammation is intimately associated with cancer initiation as well as cancer progression (106,107). Inflammation itself is a response to acute tissue damage that is initiated by a myriad of insults including infection when microorganisms intrude, exposure to toxins, ischemic injury, physical injury and other types of trauma both physical and biochemical. Once tissue damage is initiated, organisms must initiate and coordinate effective mechanisms to repair or remove damaged cells. The immune system accomplishes this in an elegant way by responding to pathogen associated molecular pattern (PAMP) molecules and/or damage associated molecular pattern (DAMP) molecules (the latter released from damaged or dying cells) via their antigen presenting cells (APC's) to initiate an immune response (108,109). According to the extended Danger model proposed by Seong/Matzinger, both PAMPs and DAMPs contain

hydrophobic regions within their structure that when exposed, can act as alarm signals to the immune system (110,111). Lipids released could also act as danger signals, due to their hydrophobicity. These lipid danger signals, either alone or possibly bound to protein DAMPs, could contribute to the inflammatory response, promote repair or immunity.

Protein DAMPs

Protein DAMPs, including HMGB1, a well-studied protein DAMP are released from damaged or dying cells, stressed cells or from areas of chronic inflammation where there may be excessive degradation of the tissue matrix (112,113). Stressors typically encountered by tumors during treatment include radiation, chemotherapeutic drugs, starvation and hypoxia. The accumulation of lactic acid in solid tumors is often thought to be caused by tumor hypoxia – a by-product of glycolysis as the tumor cells shift their mode of energy production to an anaerobic one (Warburg effect), altering the metabolic profiles of cancer cells (114).

Monocytes Are Innate Immune Effectors and Sensitive Sensors for DAMPs Found Within the Tumor Microenvironment: Possible Role of microRNAs

Myeloid cells including monocytes and macrophages are key elements which regulate tissue homeostasis and local inflammation/immunity, differentiating into various cell types in response to provocative stimuli (115,116,97). Understanding differences in response to tissue injury or damage and in particular stimuli arising from DAMPs and PAMPs, guidance for drug development to regulate the inflammatory response could be provided.

Micro-RNAs (miRNAs) are 18-22 bp long single strand RNA sequences derived from Pol II transcripts which, following processing in the nucleus and cytosol, can regulate multiple gene expression (117). miRNAs play an important role in cell differentiation, tumor progression, organogenesis and embryogenesis. Many miRNA machinery genes including Dicer, AGO1, AGO3, AGO4 are down-regulated in tumors and play a role in inflammatory cells (118-124). Various miRNAs have been identified to be involved in regulation of the cell cycle e.g. lin-4 and let-7 in control cell differentiation and proliferation, miR-14 as an apoptosis suppressor, miR-1, miR-273, lys-6, miR-181, miR-375, miR-143, and miR-196 for organogenesis. More research to identify miRNA markers for breast, lung, ovarian, cervical cancer and leukaemia is ongoing and may lead to a more refined cancer diagnostics marker in the future. Further application of miRNA as gene therapies to deliver tumor suppression as miRNA, anti-miRNA oligonucleotides (AMOs), or cholesterol conjugated AMOs, so-called antagomirs, are also in progress (125-127). It has been documented that miR 146, 181, 155 play an important role in immune regulation and inflammation. Since miRNA plays an important role in cell differentiation and proliferation affecting many cell types including hematopoietic cells, it will be useful to further understand the impact of miRNAs in the immune response in human biology. Exploring how miRNAs are involved in myeloid differentiation during the inflammatory response can help drive new strategies to limit destructive inflammation (96).

Macrophage Response To The Microenvironment

Macrophage can polarize into M1/M2 phenotype (115) or differentiate into dendritic cell 1 (DC1)/ dendritic cell 2 (DC2) or myofibroblast (116,97) in response to various stimuli. Cell surface phenotypes expressed on macrophage change significantly in response to DAMPs and PAMPs (94,95,118). Gene expression studies (89) in other cell types such as breast epithelium cell change significantly in response to hypoxia and acidosis. Small intestine submucosa (SIS) stimulates Th2 responses in animal studies (90). The Th2 associated macrophage M2 phenotype seems beneficial for wound healing.

Important questions and issues

There are several issues which need further study. These are enumerated below:

- 1-Define the Nature of DAMPs and PAMPs in the Tumor Microenvironment. This is a promising area of investigation as they may represent targets for neutralization by antibodies.
- 2-Understand the Interface Between Apoptosis and Autophagy. Since many current cancer therapeutics induce autophagy it would be sound to understand the balance in the tumor microenvironment and consider strategies to enhance apoptosis and limit autophagy.
- 3-Consider Cytokines and miRs as Potential Targets. The ability to impact on cancer will require deeper understanding of which cytokines and which miRs promote the phenotype of the disordered tumor microenvironment.

Current Clinical Evidence for Targeting Inflammation to Prevent Cancer

The complex relationship between cancer and inflammation is exemplified by the increased frequency of cancer in conditions characterized by chronic injury or inflammation. Clinically relevant examples of these relationships include chronic gastritis and gastric cancer, reflux esophagitis and esophageal cancer, cirrhosis and hepatocellular cancer, ulcerative colitis and colon cancer, and diffuse pulmonary fibrosis and lung cancer. Inflammatory infiltrates comprise a significant component of many neoplastic lesions, even in the absence of underlying inflammatory diseases. It thus appears that the at-risk organ environment presents a milieu in which carcinogenesis proceeds in complicity with the host cellular network. The inflammatory diseases that are associated with the greatest risk for cancer are characterized by abundant, deregulated and long lasting chronic inflammation. Cytokines, growth factors and mediators released in these diseases and the developing tissue microenvironment, such as IL-1 β , PGE₂, TNF- α and TGF β , have been found to have deleterious properties that pave the way for epithelial mesenchymal transition (EMT), prevent apoptosis and lead to the destruction of specific host cell-mediated immune responses against tumor antigens.

There has been significant interest in capitalizing on this knowledge of inflammatory pathways in the pathogenesis of cancer to develop effective prevention. Due to the limited progress in the development of curative therapies for most metastatic solid tumors, cancer prevention offers an alternative approach by focusing on earlier phases of carcinogenesis that may be more amenable to successful intervention. Investigations suggest that many epithelial malignancies have a long preclinical phase with molecular and histologic abnormalities that can help define populations at risk as well as targets for intervention. Chemoprevention refers to “the use of agents that can cause regression of existing preneoplastic lesions, prevent the progression of these lesions to cancer, prevent the development of new lesions” (128). The underlying concept of risk reduction is similar to the use of cholesterol-lowering drugs such as statins to reduce the risk of coronary heart disease. Bringing such interventions to clinical care, however, presents multiple scientific and logistical challenges (Table 2).

Agent selection for chemoprevention can be guided by knowledge of underlying mechanisms, epidemiology, animal models, and evidence obtained from previously conducted clinical trials (both early phase studies of chemopreventive agents and secondary endpoint analysis of studies performed in other diseases where reduced incidence of specific cancers is demonstrated) (129). For example, abundant epidemiologic data suggested that prolonged use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with decreased colon cancer incidence, while animal carcinogenesis and transgenic models provided similar experimental evidence (130). Based on this rationale, several clinical trials confirmed efficacy of NSAIDs, including the COX-2 selective agents, in reducing sporadic colorectal adenoma recurrence as well as in

reducing polyp burden in the genetic condition of familial adenomatous polyposis (FAP), which is characterized by the development of hundreds of intestinal polyps (131-137). As is the case for all diseases, however, the consideration of anti-inflammatory agents for chemoprevention requires assessment of the balance between risk of cancer and the risk of the intervention. The colorectal cancer prevention trials, while supported by a strong scientific rationale that successfully predicted positive outcomes in clinical trials, demonstrated an increase in cardiovascular events associated with the extended use of COX-2 inhibitors, resulting in the withdrawal of rofecoxib from the market and bringing into focus the importance of understanding the long-term risks of medical interventions and balancing the risks and benefits over time (129). These trials also emphasize the need for controlled trials to provide the entire clinical context for each intervention, since some toxicities do not manifest until the interventions are used for long periods by large populations, which may be missed in shorter registration clinical trials. These recent outcomes that include serious side effects identified in cancer prevention trials underscore the necessity to establish novel frameworks for agent selection for future cancer prevention clinical trials.

Due to these considerations regarding efficacy and safety and the requirement for availability of agents to use in human beings, interventions targeting inflammation for cancer prevention have thus far been primarily limited to using NSAIDs and inhaled (topical, not systemic) corticosteroids (Table 3). As discussed above, several NSAIDs have shown considerable efficacy in regressing colorectal polyps in FAP and in preventing recurrence of sporadic colorectal adenomas. However, NSAIDs have shown less promise in regressing oral leukoplakia or Barrett's esophagus (138-140). Similarly, inhaled corticosteroids did not affect the progression or regression of bronchial dysplasia in a lung cancer prevention trial, although steroid use was associated with a decrease in pulmonary nodules detected by spiral CT (141). Data is beginning to accrue from a number of early phase clinical trials using NSAIDs in a variety of other target organs, with final publications eagerly awaited. With the development of a better understanding of the nature and contribution of inflammation to carcinogenesis in various target organs, more targeted approaches to cancer prevention can be anticipated.

Potential approaches to improve the outcomes of cancer prevention clinical trials include the development of high throughput systems that will create an "individualized medicine" approach to select combinations of chemoprevention agents targeted to the specific molecular and inflammatory abnormalities in the individual at risk. The targeted prevention approach finds analogy in targeted therapies for late stage disease; patients will respond to therapies targeted to the molecular abnormalities of the tumor. Similarly, subjects at risk for cancer may demonstrate heterogeneity necessitating knowledge of the underlying inflammatory and molecular risk to specifically identify the chemopreventive agent to be selected. Regular use of aspirin appears to reduce the risk of colorectal cancers that overexpress COX-2 but not the risk of colorectal cancers with weak or absent expression of COX-2 (142). Further studies will be necessary to understand the heterogeneity of the inflammatory diseases that form the greatest risk for a variety of cancers so that specific targeted, personalized prevention can be evaluated.

The possibility of combination prevention strategies has been suggested as a means to both decrease potential toxicities and increase prevention efficacy. For example, targeting polyamine synthesis and inflammation has been shown to decrease the occurrence of the advanced and/or multiple adenomas that are most closely associated with the development of colon cancer (143). Such approaches have been successfully modeled in animal systems, but are only now entering clinical trials due to the increased logistical complexity and challenges of designing trials utilizing agents manufactured by different pharmaceutical companies.

Opportunities and challenges for cancer prevention drug development are presented by enhanced understanding of the inflammatory mechanisms of carcinogenesis and the

availability of new experimental agents. An integrative approach will be required in order to take into account the attributes of the target, the agent affecting the target and the characteristics of the subjects. It is becoming increasingly clear that effective chemoprevention will require a personalized, targeted approach. Thus, the effective development of chemopreventive agents will require a sophisticated, thorough understanding of the inflammatory processes that contribute to the diseases at-risk for carcinogenesis.

Novel Therapeutics and Clinical Trial Development to Treat Cancer

There is growing evidence that the relationship between the inflammatory process and cancer is complex. Our understanding of this relationship as it relates to both development and progression of malignancy is still limited. Further evaluation in human subjects is clearly needed if we are to truly understand whether there is therapeutic potential in targeting inflammation, or the consequences of inflammation, as an approach to treating established cancer. Several important steps need to be taken before we can know the true potential of such an approach to cancer therapy. The lack of standard nomenclature with respect to describing and grading the extent and type of inflammation within a tumor sample limits our ability to compare results from one study to another. The development of such standard criteria, as are in use for other pathologic processes, would provide consistent and accepted approaches to evaluating the number, type, and location of various inflammatory cells. Such criteria will no doubt evolve over time, and may vary from tumor type to tumor type, as data emerges relating to the clinical significance of different types and degrees of inflammation within a tumor. Nevertheless, the time is right to establish a first generation of standard criteria for describing and grading inflammation within tumors.

At this point, it is difficult to know in a particular scenario whether inflammation within an established tumor is “friend or foe”. We do not know whether inflammation is enhancing tumor-growth or providing a receptive environment for metastasis, or is serving to inhibit tumor progression. Before agents specifically designed to target inflammation within a tumor are evaluated clinically, it will be important for such basic questions to be understood. It is highly likely that the impact of inflammation will vary based on a number of factors. For example, inflammation could have a significantly different effect on the primary tumor vs metastatic disease. There are also likely to be differences in the impact of inflammation on cancer progression when comparing untreated tumors vs those being treated with traditional cytotoxic agents. That inflammation plays different roles early in oncogenesis (promoting) and later in progression (antitumor) is supported in several systems: for example, pro-inflammatory cytokine levels in patients before treatment predict the benefit of IFN immunotherapy (144). An additional issue deserving of further study will be the impact of inflammation on cancer vaccination strategies, both at the site of immunization and at the site of the effector immune response, in the tumor, and perhaps the draining lymph nodes.

Animal models have taught us much about tumor biology and identification of targets for tumor therapy. They also provide important information on mechanism of action of therapeutic agents. However, there are significant limitations to using animal models as tools to refine approaches to cancer therapy. Investigators often optimize the animal model to fit the therapy under evaluation, as opposed to optimizing the therapy to fit the animal model. This may enhance our ability to cure animals but does little to provide evidence of the likelihood of successful clinical development of the agent. Mouse models most often involve inbred animals and implanted tumors which lack not only the tumor heterogeneity but the heterogeneity of the host immune system that can have a significant impact on the inflammatory response within the tumor. Investigators prefer models where the tumor grows rapidly, thereby allowing experiments to be done relatively quickly. Such rapidly growing tumors are obviously very different from human tumors that often develop, over many years, from premalignant lesions,

grow more slowly, and have more extensive interactions with non-malignant cells within the tumor mass including inflammatory cells. Thus, while animal models are extremely useful for understanding biology and mechanisms of action of therapeutic agents, they are of limited use in fine-tuning the treatment or predicting the likelihood of clinical success of a given treatment approach. This is particularly true for therapeutic strategies targeting inflammation within tumors where the behavior of the malignant cells, and the host immune system, and how they interact, are of critical importance.

Given our limited understanding of the potential of inflammation within tumors as a target for therapy, a focus on clinical correlative studies, as opposed to design of clinical studies specifically geared towards inflammation, is likely to be most informative. Unfortunately, opportunities continue to be lost when clinical trials geared toward development of new biological therapies focus solely on clinical response rates and toxicity, with little attention played to the mechanisms and biologic changes induced by the therapeutic approach. A number of agents that would be expected to have a significant effect on inflammation within tumors are FDA approved (e.g. Bortezomib, Cytoxan and glucocorticoids) or under various stages of clinical development, yet we know little about the effect these treatments have on inflammation within tumors. Having such information would be valuable in designing subsequent studies. The hesitancy of many pharmaceutical and biotechnology companies to support correlative laboratory studies geared towards understanding mechanisms of action needs to be overcome if we are to develop strategies based on new areas of therapeutic potential such as targeting inflammatory responses in tumors. Although the timing can be challenging, correlative laboratory studies can sometimes be supplemented by non-commercial approaches to funding through the government or other sources of cancer research funding. Rigorous, well-designed smaller studies that involve sample collection, clinical evaluation, and extensive followup may well be more valuable (and certainly more practical) in this regard than the larger, multicenter, comprehensive clinical trials, especially since such larger studies often fail to collect the kind of data needed to address these questions.

Multiple factors in both the host and the malignant cells are likely to affect the impact that the malignancy has on the inflammatory response, and the impact that the inflammatory response has on the malignancy. Understanding these factors, and their relationship to treatment response, would be a central goal of correlative studies. For example, within the host, there is clearly heterogeneity in the immune response that can be evaluated genetically through the study of single nucleotide polymorphisms in immune response genes, and environmental factors such as ongoing infection that might provide ongoing signals, such as through TLRs, that impact on the inflammatory response. Within the malignant cells, signaling pathways, and production of cytokines or expression of receptors clearly play a role in how host inflammatory cells impact on the malignant cells and need to be evaluated. For example, increasing data indicates STAT 3 and STAT 5 are important in head and neck squamous cancer, prostatic adenocarcinoma, and melanoma. Understanding the effects of immunotherapy such as IFN α has on the abrogation of the immunosuppressive and anti-inflammatory effects of constitutive STAT3 activation will be very helpful in expanding our understanding why such agents mediate, or fail to mediate, an anti-tumor response. It is simply too early in our understanding of these relationships to rationally design therapeutic approaches geared specifically towards modifying these interactions in a way that will have a positive clinical impact.

Another important question related to the role of inflammation in cancer involves use of newer imaging techniques to assess response to therapy. Techniques such as position emission tomography measure cellular activity. Inflammatory cells are highly active metabolically and so can impact on our ability to correlate clinical functional imaging results with malignant activity within a tumor mass.

Conclusions

Certain treatments and targets for inflammation have come to the fore and deserve attention. In addition to the approved drugs outlined above, additional preclinical and correlative studies (145,146) may provide rationale for targeting factors and cytokines that have a clear impact on inflammation within a cancer, such as HMGB1, the receptor for advanced glycation endproducts [RAGE], and IL-1 β . Targeting these factors may decrease the incidence of cancers that develop in the setting of chronic inflammation. On the other hand, given our lack of understanding of the impact of inflammation on the progression of cancer, it would be premature to attempt a clinical trial targeting such molecules at the present time. Our limited knowledge base raises significant challenges in rationally designing clinical therapeutic strategies that target inflammation as an approach to treating established cancers. This situation should be short-lived. Our understanding of the relationship between inflammation and cancer is growing and the resulting improved knowledge base will undoubtedly allow for development of approaches to targeting inflammation in cancer that are worthy of clinical evaluation.

Acknowledgments

Source of Support: This project was funded in part by a grant from the NIH 1 P01 CA 101944-04 (Michael T. Lotze) Integrating NK and DC into Cancer Therapy National Cancer Institute. We thank the iSBTc leadership, management, and in particular Roseann Marotz (iSBTc) for her steadfast support of this project.

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Table 1

Mouse Models To Study Inflammation – Cancer Cross Talk.

Models	Pros	Cons
Xenografts	Simple, fast	Triggers massive tumor cell death, triggers an acute inflammatory response that does not occur in <i>in situ</i> tumor progression, tumors are often extremely aggressive and rarely if ever observed in humans.
Two-stage (and one stage) models of chemically-induced cancer	Easy to implement, may recapitulate some forms of human cancer	Massive mutagenesis may generate new epitopes not found in sporadic human tumors, carcinogen itself may induce a (transient) altered immune response, time-consuming, identity of activated oncogenes is not always known
Genetically engineered <i>de novo</i> mouse cancer models harboring transgenic expression of oncogenes	Fast, some models are faithful mimics of human cancer	The entire tissue is altered, potentially generating an altered microenvironment, in many cases, requires secondary genetic changes whose nature is not clear
Genetically engineered <i>de novo</i> mouse cancer models with targeted tumor suppressors.		In biallelic loss the entire tissue is altered potentially generating an altered microenvironment. Monoallelic loss variants are slow, and requires secondary genetic alterations, time consuming
Genetically-engineered mice (GEMs) with rare spontaneous activation of a dormant oncogene	Similar to human cancer	Few available strains.
Mice with tissue specific chronic inflammation (pathogens or genetic defects)	Similar to human cancer	Slow, few available strains, tumors are often heterogeneous, activated oncogenes or inactivated tumor suppressor genes that are also required are often not known

Table 2**Challenges For The Development Of Cancer Preventive Agents Targeting Inflammation.**

- Who are the subjects at greatest risk? (cohort selection)
- Appropriate agent selection (target identification)
- What are the best endpoints (surrogate endpoints, phase II vs. phase III)?
- How to incorporate the temporal changes during carcinogenesis and inflammation into clinical trials that are relatively short.
- Heterogeneity
 - Cancer - not one disease (even in the same target organ)
 - Cohort heterogeneity - pharmacogenetics, different stages of carcinogenesis, gene-environment interactions
 - Heterogeneity of the inflammatory response (in different cancers, during carcinogenesis, in different cohorts)
- Incomplete understanding of molecular pathogenesis, including inflammatory response

Table 3

Clinical Trials Targeting Inflammation For Cancer Prevention.

AGENT	TARGET	RESULT	REFERENCE
Sulindac	FAP polyp regression	↓polyps	Giardiello 1993
Sulindac	FAP new polyp development	no effect	Giardiello 2002 [135]
Celecoxib	FAP polyp regression	↓polyps	Steinbach 2000 [136]
Celecoxib	sporadic colorectal polyp recurrence	↓polyps	Bertagnolli 2006 [139]
Rofecoxib	sporadic colorectal polyp recurrence	↓polyps	Arber 2006 [140] Baron 2006 [141]
Aspirin	sporadic colorectal polyp recurrence	↓polyps	Baron 2003 Sandler 2003 [138]
Sulindac + DFMO	sporadic colorectal polyp recurrence	↓polyps	Meyskens 2008 [147]
Celecoxib	Barrett's esophagus	no effect	Heath 2007 [142]
Celecoxib	oral leukoplakia	no effect	Papadimitrakopoulou 2008 [144]
Ketorolac	oral leukoplakia	no effect	Mulshine 2004 [143]
Budesonide	bronchial dysplasia	no effect	Lam 2004 [145]

FAP=familial adenomatous polyposis